PhD Thesis

PROTEIN CHANGES OF VARIOUS TYPES OF MILK AS AFFECTED BY HIGH HYDROSTATIC PRESSURE PROCESSING

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2 NOTATION

HHP	high hydrostatic pressure
HP	high pressure
PAGE	polyacrylamide gel electrophoresis
SDS-PAGE	sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
β-Lg	beta-lactoglobulin
α-La	alfa-lactalbumin
Trp	tryptophan
Phe	phenilalanin
Glu	glutamin
Asp	asparagin
IgE	immunoglobulin E
IEF	isoelectric focusing
pI	isoelectric point
OD	optical density
Odu	optical density unit
Rf	relative front

3 INTRODUCTION

In the last few decades the concept of “minimal processing” of foods has arisen. Consumers increasingly demand foods which retain their natural, fresh-like flavour, colour and texture and contain fewer additives such as preservatives. In response to these needs minimal processing technologies have been developed. They are designed to limit the effect of processing on nutritional and sensory quality and to preserve food without the use of synthetic additives.

Traditional thermal processing techniques can be beneficial to foods in such areas as preservation and flavour formation but detrimental in damaging other sensory and nutritional properties. Minimising undesirable changes can be achieved in a number of ways, whether through more effective process control, the use of High Temperature Short Time (HTST) techniques such as aseptic processing, or newer thermal technologies such as volume heating methods. Infrared heating and dielectric methods e.g. the use of microwaves, and ohmic heating belong to the thermal minimal processing methods. Alternatives to processing by heat have been developed, ranging from irradiation to the use of pulsed electric fields. One of these novel non-thermal techniques is the application of high hydrostatic pressure for food preservation, that is currently receiving considerable attention from both researchers and producers.

High hydrostatic pressure with regard to food is in the range of 100-1000 MPa. The possibility of using high pressure to process and preserve foods has been known and studied for almost a century. Hite, a researcher of the West Virginia Agricultural Experimental Station published his findings in 1899 about “The effects of pressure on the preservation of milk”. Progress, though, has been relatively slow since it was not commercially feasible to subject foods to the pressures necessary to either preserve them or to considerably modify and improve their quality. However, in the last two decades there have been important developments in the engineering aspects of high pressure equipment so that it is now both economically and technically feasible to subject foods to the pressures deemed desirable. Although the technology is now available, or at least is rapidly becoming available, because of the cost of the equipment it is only currently applied to produce high quality, relatively expensive foods. High pressure processed foods such as jams and fruit drinks, became available in Japan around 1990. As the technology develops and becomes accepted by the consumer, the number of countries marketing and manufacturing high pressure processed foods is likely to increase. Although the capital costs may be high, production costs are not excessive, and the technology is seen to be environmentally friendly, i.e. clean and “natural” (Ledward, 1995).

Milk is an important food across the globe. Its pasteurisation is common practice in the dairy industry. Given the unassailable position of heat treatment and the diverse response of

microorganisms to pressure, particularly that milk may have a baroprotective effect for certain microorganisms, it seems unlikely that pressure will ever replace heat for the safe production of large volumes of liquid milk. Then what is the use of investigating the effects of pressure on milk and dairy products? There might be some niche products for which heat treatment could be inappropriate, and for which microbiological quality could be improved by high pressure treatment. But the main reason is that pressure brings about modifications to milk components (especially proteins), that can lead to altered functionality and the possibility of novel or improved dairy products (Needs, 2002). As a result of this it is not surprising that the dairy and food industry shows increasing interest in high pressure milk processing.

However, heat and pressure have rather different effects on the structure, interactions and properties of milk proteins. For example, during manufacture their immunoreactivity may be altered. The allergenic activity of foods may be unchanged, decreased or even increased by food processing. The molecular basis of changes in the allergenic activity is the inactivation or destruction of epitope structures or the formation of new epitopes or better access to cryptic epitopes by denaturation of the native allergen. In the model studies available in the literature the effects of heating and enzymatic digestions were investigated (Besler et al., 2001) and there is very little information about the influence of high hydrostatic pressure from this point of view.

To show possible structural changes in milk proteins polyacrylamide gel electrophoresis (SDS, native, and two-dimensional PAGE) can be used very well. Immunoblotting is a time-honoured method for the detection of immunoreactivity of proteins. Although fluorescence was one of the earliest instrumental techniques available to the analytical chemists, only recent developments in instrumentation and sample handling have made it possible for its full potential to be used in everyday analysis. Thus fluorescence is becoming one of the most promising techniques in biology, medicine, and food research as well (Deshpande, 2001). Spectrofluorometry, as a new analytical tool, was tested in the present study to observe differences between heat treated and high pressure processed milk samples, and changes in certain milk components.

4 LITERATURE SURVEY

4.1 Milk

Milk is exclusively the normal mammary gland secretion obtained from one or more milkings without either addition thereto or extraction therefrom. The natural function of milk is to nurture the young of the species. Milk has a nutritional role and hence must contain readily available sources of energy, essential fatty acids, amino acids and vitamins, all of which may be unavailable to the infant from any source other than milk. A further function of milk is to assist in combating disease, both in the mammary gland and in the infant. This is achieved by proteins such as lysozyme, peroxidase, lactoferrin and immunoglobulins. Proteinases, such as plasmin and lipases, and serum albumin or β-lactoglobulin (β-Lg), may aid digestion and nutrient absorption (Creamer, 1996).

The origin of the milk shall be indicated if it is not bovine.

Milk composition of mammalian species varies widely with reference to genetic, physiological and nutritional factors and environmental conditions (Malacarne et al., 2002).

Milk coming form cattle, goats, ewes, mares and humans was investigated in this study, their average composition is shown in Table 1.

Table 1. Average composition of different milk types (Szakály, 2001)

Component	**Concentration of the given component (%)**				
	Human milk	**Ewe's milk**	**Mare's milk**	**Goat's milk**	**Bovine milk**
Protein	**1,3**	**5,5**	**2,15**	**3,9**	**3,3**
Fat	4,5	8,2	0,6	4,0	3,8
Lactose	6,3	5,0	6,75	4,5	4,6
Minerals	0,2	0,9	0,3	0,8	0,8
Solids	12,3	19,6	9,8	13,2	12,5
Milk solids non fat	7,8	11,4	9,2	9,2	8,8
Water	87,7	80,4	90,8	86,8	87,5

Regarding their composition, human and mare's milk belong to the so called albumin milk group, while goat, ewe's and bovine milk to the casein milk group. Albumin milks are characterised by the relatively high albumin and globuline content, whereas about 80% of the total protein content are caseins in the casein milk types (Table 2.).

Table 2. Protein composition of albumin milks and casein milks (Császár and Unger, 2005)

Denomination	Protein (%)		
	Total protein	Casein	Whey protein
Casein milks			
Ewe's milk	5,35	4,3	1,05
Goat milk	3,6	2,6	1,0
Bovine milk	3,3	2,7	0,6
Albumin milks			
Human milk	1,3	0,8	0,5
Mare's milk	2,15	1,3	0,85

4.2 Milk Proteins

In milk, there are two major protein types which in bovine milk are defined by acid precipitation: the caseins, which precipitate as a group at pH 4.6, and the whey proteins, which can be subdivided into the major mammary synthesized proteins and the minor, usually blood, proteins. Each of the mammary-synthesized proteins exists in several forms, known as genetic variants, which have slightly different amino acid sequences (Creamer, 1996).

The particular sequence of amino acids in a protein determines its structure, conformation and properties. The structure of protein is categorised as primary, secondary, tertiary or even quaternary, depending on the state of spatial arrangement of polypeptide chains. The primary structure of proteins consists of a polypeptide chain of amino acid residues joined together by peptide linkages, which may also be cross-linked by disulphide bridges. The primary polypeptides in a nascent protein in an aqueous environment tend to coil in a characteristic way to form localised secondary structures, i.e. α-helix and β-pleated sheet.

They are examples of secondary structures arising from regular and periodic steric relationships. The secondary structure is stabilised by hydrogen bonds. In aqueous medium Van der Waals interactions between adjacent residues, and hydrophobic interactions between long or bulky apolar sidechains, may contribute to stabilisation of the α-helix structure. β-pleated sheets are formed where interpolypeptide chain interactions are possible, because β-turns or folds permit adjacent polypeptide chains to associate mostly via hydrogen bonding, and to a lesser extent, via hydrophobic and electrostatic interactions. The tertiary structure refers to the spatial arrangement of amino acid residues that are far apart in the linear sequence, giving rise to further coiling and folding. In a typical tertiary structure, the polypeptides are tightly folded to give a compact molecule, in which most of the polar groups of the amino acids are located on the outer surface and are hydrated. Most of the apolar groups are internal in the hydrophobic region from

which water is essentially excluded. If the protein is tightly coiled and folded into a somewhat spherical shape, it is called a globular protein. If the protein consists of long polypeptide chains which are intermolecularly linked, they are called fibrous proteins. Many globular proteins with molecular weights exceeding 50 kilodaltons are oligomeric, consisting of two or more individual (protomer)associated polypeptides. This is called quaternary structure. A protein will tend to self-associate if it contains more than 28 mol% of the particular hydrophobic amino acids, e.g, caseins (Kinsella, 1984; Goff, 1995).

Characteristics of different milk protein fractions are shown in Table 3.

Table 3. Protein fractions of bovine milk and some of their characteristics (Farrell et al., 2004)

Protein fraction	Amount in skim milk (g/l)	Genetic variants	Molecular weight (kDa)	Isoelectric point (pH)
		Caseins		
α_{s1}-casein	12-15	B	23.615	4,44-4,76
		C	23.542	…
α_{s2}-casein	3-4	A	25.226	…
β-casein	9-11	A1	24.023	…
		A2	23.983	4,83-5,07
		B	24.092	-
κ-casein	2-4	A	19.037	5,45-5,77
		B	19.006	5,3-5,8
		Whey proteins		
β-lactoglobulin	2-4	A	18.363	5,13
		B	18.277	5,13
α-lactalbumin	0,6-1,7	B	14.178	4,2-4,5
Serum albumin	0,4	A	66.399	4,7-4,9
Immunoglobulin G1	0,3-0,6	…	161.000	5,5-6,8
Immunoglobulin G2	0,05	…	150.000	7,5-8,3
Immunoglobulin A	0,01	…	385.000-417.000	
Immunoglobulin M	0,09	…	1000.000	…
Secretory components	0,02-0,1	…	63.750	…
Lactoferrin	0,02-0,1	…	76.110	8,81

4.2.1 Caseins

Caseins are phosphoproteins precipitated from raw milk at pH 4.6 at 20°C. They comprise approximately 80% of the total protein content in milk. The principal proteins of this group are

classified according to the homology of their primary structures into α_{s1}-, α_{s2}-, β- and κ-caseins (Wong et al., 1996).

Caseins are conjugated proteins, most of them with phosphate group(s) esterified to serine residues. Calcium binding by the individual caseins is proportional to the phosphate content. The conformation of caseins is similar to denatured globular proteins. The high number of proline residues in caseins causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures. The lack of tertiary structure accounts for the stability of caseins against heat denaturation, because there is very little structure to unfold. Without a tertiary structure there is considerable exposure of hydrophobic residues. This results in strong association reactions of the caseins and renders them insoluble in water. Within the group of caseins, there are several distinguishing features, based on their charge distribution and sensitivity to calcium precipitation (Wong et al., 1996; Goff, 1995; Farrell et al., 2004).

4.2.1.1 α_{s1} caseins

α_{s1} caseins have five genetic variants, A, D, B, C and E. The B variant consists of 199 amino acid residues with a calculated molecular weight of 23,614 Da. The protein contains more acidic amino acids than basic ones. It has 17 proline residues which prevent the formation of certain types of secondary structures. Three hydrophobic regions are identified that contain all the proline residues. Seven of the eight phosphate groups are located in the hydrophilic region. α_{s1} caseins are calcium sensitive, they can be precipitated at very low levels of calcium (Wong et al., 1996; Goff, 1995; Farrell et al., 2004).

4.2.1.2 α_{s2} caseins

Four variants of α_{s2} are known, A, B, C and D. The amino acid sequence of α_{s2}-CN A-11P consists of 207 residues, among them ten prolines and two cysteins, and the calculated molecular weight is 24,350 Da. Concentrated negative charges are found near N-terminus and positive charges near C-terminus. It can also be precipitated at very low levels of calcium (Wong et al., 1996; Goff, 1995; Farrell et al., 2004).

4.2.1.3 β-casein

β-casein constitutes 30-35% of the total caseins. Seven genetic variants of β-casein are known. The molecular weight of β-CN A1-5P is 23,982 Da; it is composed of 209 residues, among them 35 prolines. High negative net charge is around the N-terminal region, and the C-

terminal region is highly hydrophobic. β-casein is a very amphiphilic protein, and that's why it acts like a detergent molecule. The protein's self-association depends on temperature. It will form a large polymer at 20° C, but not at 4° C. This type of casein is less sensitive to calcium precipitation (Wong et al., 1996; Goff, 1995; Farrell et al., 2004).

4.2.1.4 κ-casein

κ-casein constitutes about 15% of the total caseins. The major casein, κ-CN B-1P contains 169 amino acid residues (twenty prolines) and its molecular weight is 19,023 Da. This protein is positioned on the outside of the casein micelle. Unlike the other caseins κ-casein is very resistant to calcium precipitation, stabilizing other caseins. Rennet cleavage at the Phe105-Met106 bond eliminates the stabilizing ability, leaving a hydrophobic portion, para-κ-casein, and a hydrophilic portion called κ-casein glycomacropeptide (GMP), or caseinomacropeptide (CMP). Cleavage of this bond is the first step in the coagulation of milk by aggregation of the casein micelles after the loss of the hydrophilic, negatively charged surface from the micelle (Farrell et al., 2004; Goff, 1995; Wong et al., 1996).

4.2.1.5 Casein Micelles

The major part of milk proteins, together with calcium phosphate, occurs in the form of large colloidal particles, the casein micelles. An average micelle contains about 10^4 caseins and its size ranges between 50 and 300 nm (Huppertz et al, 2006). Various different models have been proposed for micelle structure. One of them is the "sub-micelle model". This model suggests that casein micelles are built of roughly spherical subunits or sub-micelles. The composition of sub-micelles is variable and the size is within the range of 12-15 nm in diameter, and each sub-micelle has 20-25 casein molecules. The sub-micelles are kept together by hydrophobic interactions between proteins, and by calcium phosphate linkages. There are two main types of sub-micelles. One mainly consists of α_s- and β-caseins, hydrophobic regions buried in the center of the sub-micelle. The other type consists of α_s- and κ-caseins. The latter are more hydrophilic because of the sugar residues on them. The κ-caseins are located near the outside of the micelle with the hydrophilic part of the C-terminal end protruding from the micelle surface to form a 'hairy' layer that will avoid further aggregation of sub-micelles by steric and electrostatic repulsion. Consequently, micelles are stable, and they do not usually flocculate (Figure 1.) (Phadungath, 2005).

Another model has been evolved recently, especially from work by Carl Holt. This "internal structure" model shows a more or less spherical, highly hydrated, and fairly open particle. Holt's model of the casein micelle shows a tangled web of flexible casein networks that form a gel-like

structure with micro-granules of colloidal calcium phosphate through the casein phosphate center. The C-terminal region of κ-casein extends to form a „hairy layer" (Figure 2.). The two main features of this model are the cementing role of colloidal calcium phosphate and the surface location of hairy layer, which confers steric and/or charge stability to native casein particles (Phadungath, 2005).

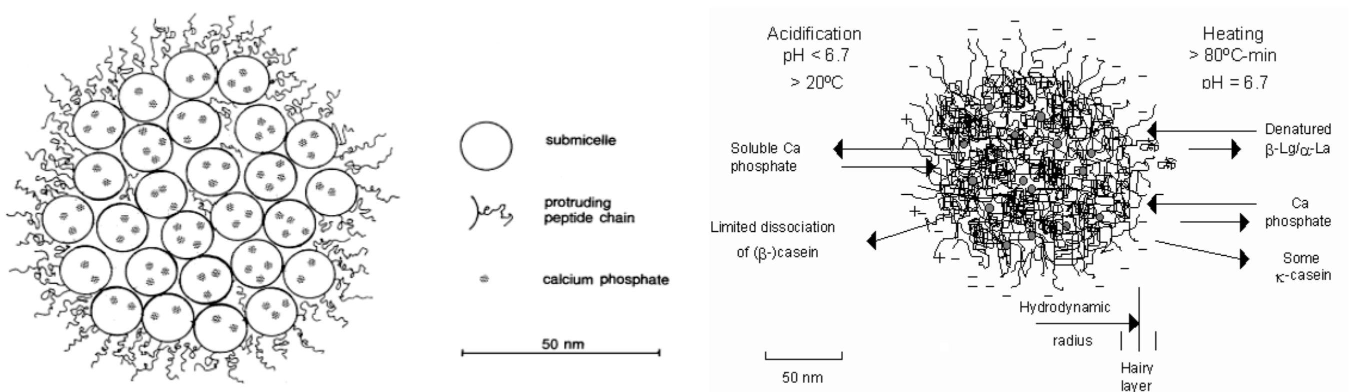


Figure 1. The sub-micelle model **Figure 2.** The internal structure model

4.2.2 Whey Proteins

The following proteins belong to the whey proteins: β-lactoglobulin (β-Lg), α-lactalbumin (α-La), lesser amounts of serum albumin, immunoglobulins, and proteose peptones. Whey proteins give 20% of total protein content in bovine milk. They are globular and are present in milk as discrete molecules with varying numbers of disulfide crosslinks. These proteins are more heat sensitive, and less sensitive to calcium than caseins. They can form disulfide linked dimers or polymers via thiol disulfide interchange e.g. with κ-casein

4.2.2.1 β-lactoglobulin

β-lactoglobulin (Fig. 3.) is the major whey protein, about 54% of whey proteins is β-lactoglobulin. Five genetic variants have been characterised. It is a globular protein with a molecular weight of 18,362 Da for variant A and 18,276 for variant B. Variant B consists of 162 amino acids. A comparison of the sequences of β-Lg in bovine, ewe's and goat milk shows, that the three proteins are highly homologous. They contain two intrachain disulfides and one sulfhydryl group. Horse β-Lg A has no free thiols, because cisteine is replaced by a tyrosine residue. Variant B consists of 166 amino acids (Wong et al., 1996).

The secondary structure of bovine β-Lg is 15% α-helix, 50% β-sheet and 15-20% reverse turn. The protein is a typical lipocalin whose structure thus contains a β-barrel with eight antiparallel β-strands, labelled A–H and a three-turn α-helix that lies parallel to three of the β

strands. Strands A–D form one surface of the barrel while strands E–H form the other. A significant feature in all lipocalins is the bend in strand A that allows it to interact with strand H. The three-turn-α-helix follows strand H and lies on the outer surface of the barrel between the C-terminal end of the A strand and the H strand. A ninth β-strand, I, antiparallel to the first strand, A, and on the other side of H, is used in dimer formation (Kontopidis et al., 2004).

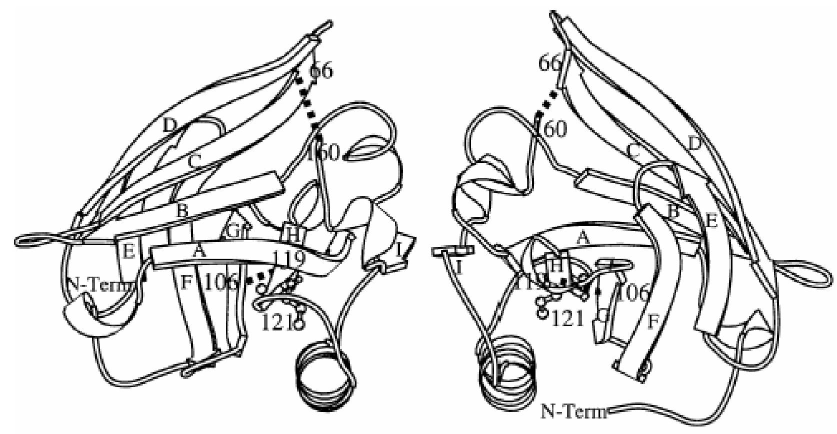


Figure 3. Structure of β-lactoglobulin (Qi et al., 1997)

The molecule contains two disulfide bonds, which are found between cisteins 106. and 19., and the cisteins 6. and 160., respectively. There is one free sulfhydril group in β-Lg, but there is no phosphorus present in this protein.

β-Lg is very acid stable. It is generally in dimer form at the isoelectric pH of 5.2 and alkaline pH range. Bovine β-Lg denatures at temperatures above 65°C at pH 6.7, typically at 70.4±0.5°C, followed by aggregation. Denaturation temperature of β-Lg depends on pH. It is most heat sensitive near pH 4.0 and most stable at pH 6.0.

Temperature affects the three dimensional structure of β-Lg. Although β-Lg is found mainly in the dimer form in milk, monomers appear when temperature is increased up to 65°C. Critical conformational change occurs around 63°C, where there is 19% net reduction in the β-sheet content, as shown by circular dichroism (Prabakaran, Damodaran, 1997). This reduction in β-sheet content seems to be critical for initiating sulfhydryl disulfide-induced aggregation. Above this temperature, unfolding of β-Lg structure leads to irreversible denaturation in the following order: D-E strand (55-60°C); C-D strand and α-helix (60-65°C); A-B, A-I and E-F strands (65- 70°C); and A-H, B-C and F-G strands (75-80°C). Thermal unfolding of β-Lg is almost complete at 80°C except for the G-H pair of disulfide-linked strands which are the most heat-resistant feature of the structure (Edwards et al., 2002; Doucet, 2004).

β-Lg was found to bind retinol and enhance its fluorescence. One molecule of retinol is bound per β-Lg monomer. Binding of retinol by β-Lg occurs in the interior of the hydrophobic

barrel with tryptophan19 at the bottom of the calyx interacting with the β-ionine ring of the retinol molecule (Wong et al., 1996).

β-Lg is is one of those milk proteins that are responsible for milk protein intolerance or allergy in humans (Bonomi et al., 2003; Clement et al., 2002).

4.2.2.2 α-lactalbumin

Bovine α-La (Fig. 4.) is a small globular protein that is relatively stable. It constitutes 21% of whey proteins. Its genetic variant A has a molecular weight of 14,147 Da. Variant B has a molecular weight of 14,175 Da. α-La is composed of 123 amino acid residues. The molecule has an ellipsoid shape with a deep cleft dividing the protein in two parts. Four helices form one side of the cleft and two β-sheets together with a loop-like chain make up the other one. Four disulfide bonds make this protein relatively heat stable.

α-La was found to be a cofactor in lactose synthesis and the concentrations of this protein and of lactose in milk are correlated. It is a strong binder of calcium and other ions, including Zn(II), Mn(II), Cd(II), Cu(II), and Al(III), and changes conformation markedly on calcium binding (Wong et al., 1996). One interesting feature of α-La is that it seems to exist in three different structures: the calcium-bound, the calcium-free and the low pH or A form. Recently, this latter form has been studied intensely as it may constitute a new protein structure. This 'molten globule' structure may be intermediate between the native and denatured forms of the protein (Creamer, MacGibbon, 1996).

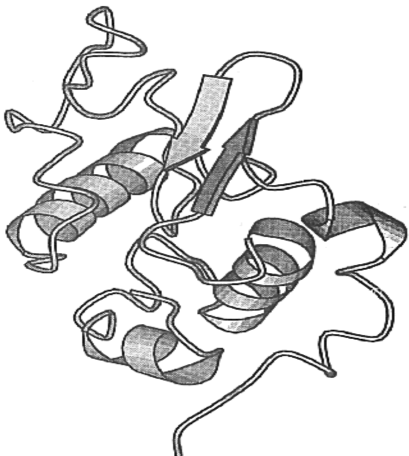


Figure 4. The structure of α-lactalbumin

4.2.3 Tryptophan in Milk Proteins

The folding of a polypeptide chain to form a relatively compact globular protein inevitably results in the burial of certain amino acid residues from the external, aqueous environment. Other

residues, either by choice or chance, will lie on the surface, exposed to the polar solvent. A strategy often employed in studying the solution structure of proteins is to map out those residues which are exposed, versus those which are buried. Since most proteins contain a relatively small number of tryptophanyl residues, this amino acid has received considerable attention in such topographical studies (Puyol et al., 1991).

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan (Trp) (Fig. 5.) residues with some emissions due to tyrosine and phenylalanine. Trp is an important derivative of indole, whose photophysical properties have been extensively studied because of its importance in fluorescence investigations of proteins (Royer, 1995).

Typically, tryptophan has a wavelength of maximum absorption of 280 nm and an emission peak, that is solvatochromic, ranging from ca. 300 to 350 nm depending on the polarity of the local environment. Hence, protein fluorescence may be used as a diagnostic of the conformational state of a protein. Trp has much stronger fluorescence and higher quantum yield than the other two aromatic amino acids (Tyr and Phe). The intensity, quantum yield, and wavelength of maximum fluorescence emission of Trp is very solvent dependent. The fluorescence spectrum shifts to shorter wavelength and the intensity of fluorescence increases as the polarity of the solvent surrounding the Trp residue decreases. Trp residues that are buried in the hydrophobic core of proteins can have spectra that are shifted by 10 to 20 nm compared to Trp-s on the surface of the protein. Trp fluorescence can be quenched by neighbouring protonated acidic groups such as Asp or Glu.

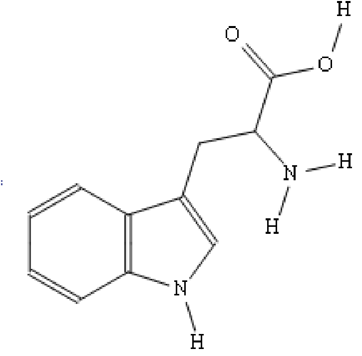


Figure 5. Chemical structure of tryptophan

Also, energy transfer between Trp and the other fluorescent amino acids is possible, which would affect the analysis. In addition, Trp is a relatively rare amino acid; many proteins contain only one or a few Trp residues. Therefore, Trp fluorescence can be a very sensitive indicator of

the conformational state of individual Trp residues. The advantage compared to extrinsic probes is that the protein itself does not change. In pratice the use of intrinsic fluorescence for the study of protein conformation is in practice limited to cases with few (or perhaps only one) Trp residues, since each experience is conducted in a different local environment, which gives rise to different emission spectra (Mocz, 1999).

The quantum yields for all three aromatic amino acids decrase when they are incorporated into a polypeptide chain. The fluorescence of the aromatic residues varies in somewhat unpredictable manner in various proteins. Comparing to the unfolded state, the quantum yield may be either incraesed or decreased by the folding. Accordingly, a folded protein can have either greater or less fluorescence than the unfolded form. The intensity of fluorescence in itself is not very informative. The magnitude of intensity, however, can serve as a probe of perturbations of the folded state. The wavelenght of the emitted light is a better indicator of the environment of the fluorophore. Trp residues that are exposed to water have maximal fluorescence at a wavelength of about 340-350 nm, whereas totally buried residues fluoresce at about 330 nm.

As for caseins, there are two Trp residues in α_{s1}-CN B-8P and α_{s2}-CN A-11P, and one Trp residue in β-CN A1-5P and κ-CN B-1P. Among whey proteins β-Lg has two tryptophanyl residues, and α-La has three (Wong et al., 1996). Papiz and co-workers (1986) noted about the crystal structure of β-Lg that Trp-19 is at the bottom of the central hydrophobic calyx of the protein, while Trp-61 is part of an external loop. The intrinsic fluorescence of β-Lg is therefore almost exclusively attributed to Trp-19, positioned in a more apolar environment than Trp-61. β-Lg exhibits structural and binding properties, that vary widely, depending on the medium. These properties of β-Lg are reflected in fluorescence intensities, steady-state anisotropies and phase lifetimes of β-Lg Trp residues.

4.2.4 Retinol in Milk

Retinol (Fig. 6.), the dietary form of vitamin A_1, is a yellow, fat-soluble, antioxidant vitamin. It belongs to the family of chemical compounds known as retinoids.

Figure 6. Structure of retinol.

Retinol (about 1µmol/l, in bovine milk) is located in the core and in the membrane of the fat globules. Due to its four conjugated double bonds, retinol is a good fluorescent probe with excitation and emission wavelengths at about 330 and 450 nm, respectively. The fluorescence properties of retinol change as a function of the environment. A very weak fluorescence is observed in aqueous solutions of retinol, but its quantum yield is drastically enhanced in an apolar environment (Dufour, Riaublanc, 1997).

4.2.5 Immunoreactivity of Milk Proteins

Allergy is a hypersensitivity reaction to macromolecules (generally proteins). They are commonly mediated by a specific class of antibodies, known as immunoglobulin E (IgE), which is normally generated as part of immune reactions to parasitic infections. But for reasons that are only partly understood, they can also be generated after exposure to environmental agents, such as pollen, dust, and foods. Only about eight types of foods are reponsible for causing the majority of food allergies, cow's milk being one of them (Mills, Breiteneder, 2005). Cow's milk allergy has become a common health condition in early childhood, its prevalence ranging from 1.6 to 2.8% in children younger than 2 years of age. Although most infants with IgE mediated cow's milk allergy outgrow their sensitivity by they third year, 15% retain their sensitivity into their second decade (Natale et al., 2004). The major allergens in milk are caseins, whey proteins β-Lg, α-La and serum albumine (Besler et al., 2001), but most cow's milk proteins are potential allergens, even proteins that occur in very low concentrations (Wal, 2002).

There are antigen determinant groups (epitopes) on the surface of allergen proteins that enable linkage with the IgE antibody. There are both conformational and linear epitopes widely spread all along the protein molecules. They may be short fragments located in hydrophobic parts of the molecule which comprise highly conserved sequences responsible for IgE cross reactivity with corresponding milk proteins of other mammals, including human beinngs (Wal, 2002). Structural studies of milk allergens have revealed that the conformations of the allergenic loops are very similar in α-La and β-Lg. It is suggesting a characteristic conformation for the

allergic sites in the proteins (Sharma, 2000). At the same time no specific structure nor function is associated with allergenicity of cow's milk proteins. Variability and heterogeneity of the human IgE response preclude the feasibility of predicting the allergenic potential of any cow's milk protein or its fragment.

Patients suffering from cow's milk allergy very often show cross-reactivity to goat's and ewe's milk, respectively. This is not surprising, since α_{s1}- and α_{s2}-caseins of these animals share 87% to 98% identical amino acids. This biochemical similarity is connected to the same phylogenetic origin of these species (Bellioni-Businco et al., 1999). The amino acid composition of proteins in mare's milk widely differs from that of the above mentioned animals. This explains very likely why cross-allergy occurs less frequently in reaction to mare's milk (Businco et al., 2000).

4.2.6 Microbiology of Milk

In addition to being a nutritious food for humans, milk provides a favourable environment for the growth of microorganisms. The temperature of freshly drawn milk is about 38°C. Yeasts, moulds and a broad spectrum of bacteria can grow in milk, particularly at temperatures above 16°C.

Microbes can enter milk via the cow, air, feedstuffs, milk handling equipment and the milker. Once microorganisms get into the milk their numbers increase rapidly. The initial bacterial count of milk may range from less than 1000 cells/ml to 10^6/ml. High counts (more than 10^5/ml) are evidence of poor production hygiene (International Livestock Research Institute, 2008). Owing to the different properties of the various bacteria the question of contamination is not only limited to the total number of bacteria but in many respects even more to the bacteria species. Storage of milk at low temperatures will also result in a change in the microbial balance in favour of those multiplying at low temperature, such as the psychrotrophic bacteria. The most common Gram negative psychrotrophic bacteria belong to *Pseudomonas, Alcaligenes, Achromobacter* and *Flavobacterium* and 60% of the Gram positive non-sporeforming psychrotrophic bacteria pertain to the genus *Arthrobacter*.

As *Pseudomonas* strains are often rather proteolytic and lipolytic, the milk will easily deteriorate even though it is stored at a rather low temperature. Proteolysis of the milk will not only result in off flavour but also in a smaller yield when the milk is used for cheese production.

Lipolysis causes the milk to become rancid in a very short time, a flavour which is easily transferred to various milk products such as butter and cheese.

As special requirements, milk must contain only a few thermoduric bacteria if it is to be used for the production of liquid milk or milk powder, since these bacteria survive the normal heat treatment of milk for this purpose.

If the milk is used for cheese production, care must be taken to prevent the milk being contaminated with gas-producing bacteria since they may result in swelling of the cheese. Coliform bacteria are destroyed by the normal heat treatment of milk but recontamination is rather common.

More severe is contamination with anaerobic sporeformers since the spores are not destroyed by the heat treatment (Cross, Overby, 1988).

Bacterial types commonly associated with milk are given in Table 4.

Table 4. Bacterial types commonly associated with milk.

Pseudomonas	Spoilage
Brucella	Pathogenic
Enterobacteriaceae	Pathogenic and spoilage
Staphylococci	
Staphylococcus aureus	Pathogenic
Streptococci	
S. agalactiae	Pathogenic
S. thermophilus	Acid fermentation
S. lactis	Acid fermentation
S. lactis-diacetylactis	Flavour production
S. cremoris	Acid fermentation
Leuconostoc lactis	Acid fermentation
Leuconostoc lactis	Acid fermentation
Lactobacilli	
L. lactis	Acid production
L. bulgaricus	Acid production
L. acidophilus	Acid production
Propionibacterium	Acid production
Mycobacterium tuberculosis	Pathogenic

Natural souring of milk may be advantageous. The low pH retards growth of lipolytic and proteolytic bacteria. The acidity of the milk also inhibits the growth of pathogens. It does not, however, retard the growth of moulds.

Fermented milk is used to make many products, e.g. yoghurt, sour cream, ripened buttermilk and cheese. These products provide ways of preserving milk and are also pleasant to consume.

They are produced by the action of fermentative bacteria on lactose and are more readily digested than fresh milk (International Livestock Research Institute, 2008).

4.3 High Pressure Processing

4.3.1 Short History

Most likely Certes was the first researcher to publish data in 1883 about the effects of high hydrostatic pressure (HHP) on organisms. He found viable bacteria in water obtained from 5100 m depth. HHP treatment of food was applied for the first time by Bert Hite in 1899 (Knorr, 1995). He inoculated milk samples with pure cultures of “anthrax, typhoid, tuberculosis, *Proteus vulgaris,* and bubonic plague”. Some of each of the organisms survived pressure treatment and despite of a catastrophic pressure vessel failure involving typhoid, so too did the investigating workers the accident (Johnston, 1995). Chlopin and Tamman (1903) used pressures of about 300 MPa and reported, that some of the tested micoorganisms changed under high pressure into “a condition of faint from which they do not recover until some time”.

After a long pause in 1965 appeared the next publication by Timson and Short. They showed that spores had higher resistance to pressure than vegetative cells. In 1970 Gould and Sale demonstrated that pressure induced germination of spores. After more than one decade high pressure research on food systems was resumed in 1982 by Hoover and his co-workers at the University of Delaware. At Kyoto University food-related high pressure activities were begun in 1989, and the Japanese Society for High Pressure was formed. The first commercial products preserved by high pressure appeared on the Japanese market as early as 1990. Since then academic and industrial research and interest in the application of high pressure is uninterrupted, first of all in Japan, the U.S.A. and Europe (Knorr, 1995; Farkas, Hoover, 2000).

The main reason of the long intermission in the field of high pressure investigation was the lack of appropriate equipment. High pressure technology has depended largely on the development of guns or cannon. High pressure equipment has to be designed to effectively generate and hold the desired pressure while remaining controllable. Operational safety (“leak before fracture”), protection of personnel must be ensured, and an acceptable economic lifetime must be achieved (Crossland, 1995). The status of technology today is such that capacity, operating, process control, and safety requirements can readily be met. Commercial high-pressure food processing must meet specific requirements with regard to sanitation and cleaning, material handling, package design, and operational cost effectiveness (Mertens, Deplace, 1993).

4.3.2 General Overview of High Hydrostatic Pressure

High pressure (HP) has been used effectively for decades in several industrial branches, such as the oil and metallurgical industries and in the production of special ceramics and plastics.

According to Pascal's law, pressure acts instantly, isostatically and homogenously, independently of the size and shape of the material. In high pressure treatment of foods, pressures between 100 and 1,000 MPa are used. This is higher than pressures present in deep sea. During HHP treatment the food packed in a flexible packaging material is put in a high pressure cylindrical vessel where it is surrounded by a non-compressible pressure-transmitting medium, usually water. The transmitting medium is pressurized up to the treatment pressure. This pressure is kept constant from a few minutes to multiples of times 10 minutes.

It has been established for both solid and liquid foods with moderate or high water content that the pressure is equal at each point of the treated product to the pressure of the transmitting medium (Mermelstein, 1998).

In the food industry the main field of application of HHP is food preservation. Food spoilage is very often caused by microorganisms and biochemical processes catalyzed by ezymes. With HHP a great part of microorganisms can be destroyed and most of the enzymes can be inactivated (Mertens, Deplace, 1993). Using HHP treatment, undesirable changes and thermal degradation of heat-sensitive food components can be avoided, a major advantage. The treatment is effective at ambient or moderate temperatures. Tests show that this treatment affects only the non-covalent bonds (i. e. hydrogen, ionic and hydrophobic) bonds, and impacts taste, colour and nutritional value of foods to a negligible degree. Thermal treatment, on the other hand, changes the covalent bonds and significant changes are also observed in food components. Depending on the kind of food the effects can be beneficial or undesirable (colour, texture, structure etc.). In general, components with low molecular weight remain intact while macromolecules (proteins, complex carbohydrates) undergo changes (Datta, Deeth, 1999).

HHP also affects biochemical reactions. Pressure reduces the size of the molecules and promotes bond formation between side-chains (Hoover et al., 1989). Protein molecules are denatured under high pressure. This is a complex phenomenon: it depends on the structure of the proteins, the extent of the pressure, the temperature and the pH (Zamyatnin, 1972; Hinrichs and Rademacher, 2002). The effect of HHP on microorganisms depends on the composition of the foodstuffs and the physiological condition of the microorganisms.

HHP also affects the morphology of microorganisms. Survival of the microorganisms depends on the extent of pressure, holding time and temperature, composition of the food and the condition and growth phase of microbes (Patterson et al., 1995). Pressures between 300-600 MPa inactivate yeasts, moulds and most of the vegetative bacteria. Bacterial spores can be

destroyed substantially only with pressures higher than 1000 MPa. Pressures between 50 and 300 MPa may even stimulate spore germination. It is known from the literature, how differently pathogens react to HHP in milk. Selected data are shown in Table 5 (Koncz et al., 2007).

Table 5. Effect of high hydrostatic pressure on foodborne microorganisms in milk according to data from the literature (Koncz et al., 2007)

Author	Treatment		Foodborne microorganism	
	Pressure (MPa)	Time (min)	Species	Lethality
Patterson et. al. (1995)	600	10	*E. coli* *S. aureus*	-1 -1.5
Erkmen & Karatas (1997)	250 350	5 4	*S. aureus*	-1.5 - 5
Gervilla et.al. (1997)	350 400	10 10	*Listeria innocua 910 (non pat.)*	-1 -4
Oliveira et. al. (1999)	500 500	10 10	*Salmonella* *E. coli*	- 6-7 - 6-7
Rademacher et. al. (2001)	600 600	20 8	*E. coli* *L. monocytogenes*	-7 -7

The advantages of HHP processing can be summarised in the followings:

- High retention of colour, aroma, and nutritional value;
- Potential to form novel texture;
- The food is pressurized in packaged form (no re-contamination);
- Positive consumer acceptance.

Perhaps the greatest hindrance of the broader application of HHP in food industry is the high investment costs of the equipment, that can be compensated by the smaller energy and running costs. Other problems that should be solved on the introduction of this technology: continuous processing is difficult; destruction of spores needs combined treatment; enzyme inactivation is not complete; flexible packaging materials are necessary; food has to contain min. 40% water to achieve antimicrobial effect. Legislation is still lacking, although according to the “Novel Food Regulation” (EC No.258/97) food treated at pressures higher than 150 MPa can be considered as “novel” food (Behsilian et al., 2003).

There are still some other aspects to be taken into consideration regarding commercial HHP processing, and all in all the general view is among the experts of the food industry, that HHP technology is too risky at the moment for the major companies, and its users tend to be small or medium-size food producers (Corkindale, 2006).

Fifty-five companies used HHP technology in 2005, that meant about 90 pieces of industrial-scale equipment. Most of them work in America (U.S.A., Mexico, Canada: 56 pcs). In Europe 19

HHP equipment have been installed, and 14 pcs in Asia. The total production amounted to 100-120 thousand tons in that year.

However, spreading of HHP technology is supposed to grow gradually, and in spite of the higher product prices once consumers have tried HHP food, they keep choosing it, no matter what it costs.

4.3.3 High Pressure Equipment

A schematic diagram of basic equipment design used for HHP processing is presented in Fig.7.

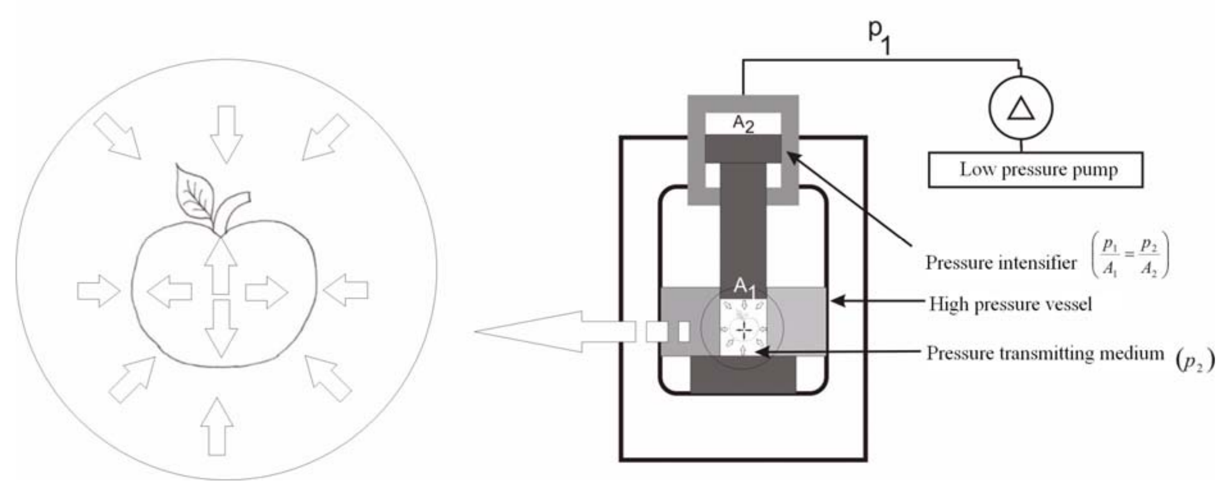


Figure 7. Schematic diagram of basic equipment design for high pressure processing of foods (Barta, 2007)

A typical HHP system consists of four main parts: a high pressure vessel and its closure, a pressure-generating system, a temperature-control device and a material-handling system (Mertens, Deplace, 1993; Mertens, 1995). The pressure vessel is usually a forged monolithic cylinder made of low-alloy steel of high tensile strength. The wall thickness is determined by the maximum working pressure, the vessel diameter and the number of cycles the vessel is designed to perform; this thickness can be reduced by using multi-layer, wire reinforced or other pre-stressed designs (Mertens, Deplace, 1993). Once loaded and closed, the vessel is filled with a pressure-transmitting medium. In food processing generally potable water or ethanol are used (Myllymäki, 1996). Air must be removed from the vessel, by compressing or heating the medium, before pressure is generated (Deplace, 1995). In the food industry, vessels with a volume of several thousand litres are used, with typical operating pressures in the 100 MPa – 500

MPa range, and holding times of about 5–10 minutes (Myllymäki, 1996). Laboratory-scale HHP equipment capable of reaching pressures up to 1000 MPa is also available.

4.3.4 Principles of High Pressure Processing

Pressure and temperature determine many properties of inorganic and organic substances. In food preservation, thermal processing is commonplace. If, however, a substance is exposed to increasing pressure, many changes will occur, especially at pressures of several hundred MPa (Buchheim, Prokopek, 1992). The behaviour of biological macromolecules under pressure is important for understanding the effects of HHP on milk. Under pressure, biomolecules obey the Le Chatelier-Braun principle, i.e., whenever stress is applied to a system in equilibrium, the system will react so as to counteract the applied stress; thus, reactions that result in reduced volume will be triggered under HHP. Such reactions may result in inactivation of microorganisms or enzymes and in textural changes in foods (Balci, Wilbey, 1999).

If the conditions for equilibrium or isokineticity are plotted against temperature and pressure, a stability phase diagram is obtained with an elliptical shape. Of particular interest in food processing are effects of HHP on proteins. Figure 8. shows a schematic pressure-temperature diagram of proteins. Proteins can be denatured using heat, pressure, and low temperatures.

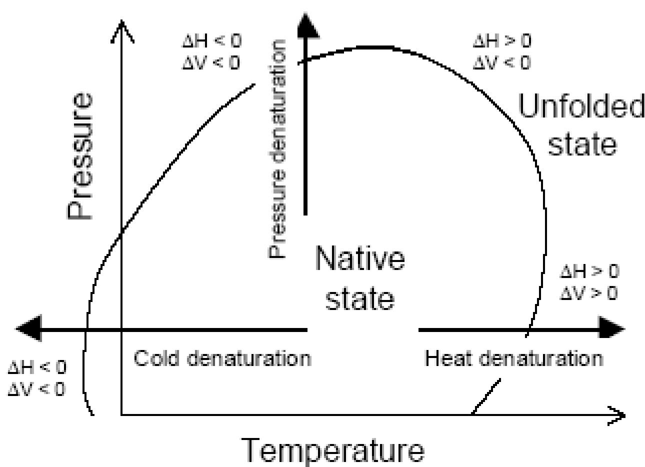


Figure 8. Typical phase transition curve of proteins in the *pT*-diagram. The relation between heat-, cold and pressure-denaturation of proteins is presented by the sign of enthalpy changes (ΔH) and volume changes (ΔV) (Heremans, 2002)

Denaturation of single-chain proteins may be regarded as a two-component system, where the native and denatured forms of protein are interchanging. From Fig. 8. it is apparent that denaturation temperature rises initially as the pressure rises. At maximum transition temperature

the sign of volume (ΔV) changes. From this point on the the protein denatures at lower temperatures at the given pressure. At the maximal transition temperature the sign of entropy (ΔS) changes and from this point on the protein denatures at lower pressures at the given temperature.

4.3.4.1 The Two-state Model and the Phase Transition

The folding–denaturing transition in proteins is a highly cooperative process. In certain cases, as a rule for smaller proteins, it suffices to describe this transition within a two-state approach involving the native state N, and the denatured state D, only. All those states are associated here in which the protein is working with the native state N, and all those states in which the protein is not working with the denatured state D. Despite the large structural manifolds involved, the two-state approach seems to work well in case that the two phase space areas can be lumped together to form two effective states. A prerequisite for this kind of state lumping is that thermodynamic equilibrium is established, an assumption which is itself quite severe and not always easily proved.

Provided that all these assumptions hold, the simplest approach to model protein stability is to consider the folding–denaturing transition as a phase transition. If in the D-N two-phase system the phases are in equilibrium, while material of a certain weight transfers from one phase to the other, then the Clausius-Clapeyron equation is valid:

$$dP/dT=\Delta S/\Delta V \qquad \textbf{Equation 1.}$$

Note that Eq. (1.) is an immediate consequence of the condition for the phase boundary, $\Delta G=0$. ΔS and ΔV are the entropy and volume changes associated with the transition. Both quantities depend on the actual pressure P, and temperature T, where the transition takes place. The boundaries of the stability phase diagram, i.e. the area in a pressure–temperature plane where the protein is stable in its native state, can then be determined from a solution of Eq. (1.). This equation is readily solved by resorting to a further approximation.

In Eq. (1.) ΔS, and ΔV represent the differences in entropy, and volume, respectively, in the individual phases. These quantities are in close relation to the specific heat capacity and the thermal expansion. These are system parameters which we assume to be well defined, i.e. to be roughly independent on pressure and temperature as mentioned above. If so, ΔS and ΔV in Eq. (1.) depend only linearly on T and P, and, hence, the equation can easily be integrated. The result is a general 2nd order curve in P and T whose shape may be elliptic, parabolic or hyperbolic:

$$aP^2 + bT^2 + 2cPT + 2fP + 2gT + const = 0$$ **Equation 2.**

4.3.4.2 Stability Against Temperature

During the temperature-induced denaturing transition, a protein changes from a rather well-organized structure into a random coil-like structure in which the hydrophobic amino acids come into contact with water. As a consequence, water forms locally ordered structures around the hydrophobic molecules, the so-called iceberg. These local structures are characterized by a low entropy as well as by a low enthalpy due to the wellaligned hydrogen bonds. The change of the specific heat, $\Delta C_P = Cp_D - Cp_N$, associated with the transition, is generally assumed to be positive, in agreement with the experimental findings. The difference in enthalpy, ΔH, between the native and the denatured state increases as the temperature is raised, according to $\Delta H(T) = H(T_1) + \Delta C_P[T-T_1]$. At the same time, the respective difference in entropy, ΔS, increases as well, since the ordered solvent structures melt away. At some critical temperature $T = T_0$, the enthalpic term, ΔH, and the entropic term, -TΔS, cancel, rendering a free energy change ΔG of zero. At this temperature, namely at $T = T_0$, the transition to the denatured state takes place because it is energetically more favorable.

The same arguments can be used to understand, on a qualitative level, the phenomenon of cold denaturation: Lowering the temperature decreases the enthalpic term (note that, in this case $T < T1$) so that it eventually becomes negative and may compensate the entropy term, TΔS, which is positive due to a decreasing entropy. The actual transition temperatures into the denatured state depend of course on pressure: High pressure at low temperature may destabilize the locally ordered structures („iceberg”) because it counteracts an optimum alignment of the hydrogen bonds.

4.3.4.3 Stability Against Pressure

The free energy change associated with protein denaturation, becomes lower as pressure is increased, at least above some threshold pressure. We can use similar arguments as above, namely that $\Delta G(T) = \Delta G(T_1) + \Delta V[P-P_1]$. $\Delta V = V_D - V_N$ is the volume change in going from the native to the denatured state. As a rule, ΔV is negative because the structure of the native state has voids, for instance in the protein pockets, which are squeezed away in the denatured state so that its volume is smaller and, hence, the transition into the denatured state becomes favored under high pressure. Increasing the pressure up to some critical level $P=P_0$, the protein may eventually cross the boundary $\Delta G=0$, and the transition to the denatured state takes place.

The respective transition at low pressure is less straightforwardly to understand. First of all, we note that, in a large temperature range, the low pressure denaturation regime would require negative pressure, a condition which has, so far, not been realized experimentally. There is indeed a temperature range in which high pressure leads to a stabilization of the native state, and, consequently, low pressure to a destabiliation associated with denaturation. At rather high pressure (i.e. outside this range) the denatured state is far from being a random coil state. It is plausible that unfolding to a random coil against high pressure is severely hindered. Instead, the high pressure denatured state is still kind of a globular state where the voids in the protein are squeezed to a high degree so that $V_D < V_N$. On the other hand, in the lower pressure range and at sufficiently high temperature, unfolding to a random coillike state is still possible. Accordingly, the protein acquires a larger surface and, concomitantly, a larger volume. In addition, compression is much harder than in the native state because the compressible voids have vanished and the hydration shell is harder to compress than bulk water due to the ordered structures induced by the hydrophobic amino acids (Scharnagl et al., 2005).

4.3.5 Effect of High Hydrostatic Pressure on Proteins, with Special Regard to Milk Proteins

In their native state, proteins are stabilised by covalent bonds (including disulphide bridges) plus electrostatic interactions (ion pairs, polar groups), hydrogen bridges and hydrophobic interactions. Covalent bonds are almost unaffected by HHP, at least at relatively low temperatures (0–40°C), and so the primary structure of proteins remains intact during HHP treatment (Mozhaev et al., 1994). High pressure affects:

a.) the quaternary structure (e.g. through hydrophobic interactions);

b.) the tertiary structure (e.g. through reversible unfolding);

c.) the secondary structure (e.g. through irreversible unfolding) (Balci and Wilbey, 1999).

Stabilising hydrogen bonds are enhanced at low pressures and ruptured only at very high pressures. Significant changes to the tertiary structure of proteins, which is maintained chiefly by hydrophobic and ionic interactions, are observed at >200 MPa (Hendrickx et al., 1998). Multimeric proteins, held together by non-covalent bonds, dissociate at relatively low pressures (~150 MPa), thereby disrupting quaternary structures. The exposure of protein surfaces, that formerly interacted with each other, to a solvent (hydrophobic solvation), results in the binding of water molecules, thereby reducing the volume of the system; thus, increasing pressure moves

the equilibrium between monomeric and multimeric states of proteins towards monomerisation (Gross, Jaenicke, 1994; Hendrickx et al., 1998).

Exposed to pressures above 400 MPa most of the proteins denature. Sensitivity to pressure or temperature varies with the type of bonds maintaining the structure. Measurements showed that structures with β-sheets are more stable against pressure than those with α-helices. The former is nearly incompressible while the latter can be deformed more easily. Oligomeric proteins dissociate to subunits while volume decreases. After dissociation subunits may reaggregate or denature. At pressures above 200 MPa chains begin to unfold and subunits of dissociated oligomers start reassociating. However, small molecules that have little secondary, tertiary and quaternary structure, such as amino acids, vitamins, flavour and aroma components, remain unaffected (Balci, Wilbey, 1999).

4.3.5.1 Effect of High Hydrostatic Pressure on Whey proteins

The behaviour of whey proteins under HHP is particularly important for milk and dairy products.

Johnston et al. (1992) were among the first researchers, who investigated the effects of HHP on whey proteins. The authors found that the amount of non-casein nitrogen decreased in milk serum with increasing pressure, that suggested denaturation and insolubilisation of whey proteins.

It was published in several studies that β-Lg is more sensitive to pressure than α-La. Denaturation of whey proteins is usually determined by a loss in solubility at pH 4,6. With this method α-La was denatured at pressures higher than 400 MPa, and β-Lg at pressures higher than 100 MPa. The higher barostability of α-La is related to its more rigid molecular structure because there are four intra-molecular disulphide bonds in the protein, while in β-Lg there are only two. Besides, β-Lg contains a free sulphydril-group which can participate in sulphydril oxydation or sulphydril-disulphide interchange reactions (López-Fandiño et al., 1996; Hinrichs et al., 1996; Felipe et al., 1997; López-Fandiño, 1998; López-Fandiño, Olano, 1998; Garcia-Risco et al., 2000; Scollard et al., 2000; Huppertz et al., 2004; Hinrichs, Rademacher 2004; Huppertz et al., 2004b; Zobrist et al., 2005). After treatment at 400 MPa denaturation of β-Lg reached 70-80%. At higher pressures, at 400-800 MPa, relatively little further denaturation occurs (Scollard, 2000).

The extent of HHP-induced denaturation of α-La and β-Lg increases with increasing holding time, temperature, and pH of milk (López-Fandiño et al., 1996; Felipe et al., 1997; López-Fandiño, 1998; López-Fandiño, Olano, 1998; Garcia-Risco et al., 2000; Scollard et al., 2000;

Huppertz et al., 2004; Hinrichs, Rademacher 2004; Huppertz et al., 2004b; Gaucheron et al., 1997; Arias et al., 2000).

Under HHP β-Lg unfolds and thus its free sulphydril group gets exposed. During HHP treatment of milk, denatured β-Lg may form small aggregates (Felipe et al., 1997) or interact with casein micelles (Needs et al., 2000a; Scollard et al., 2000). Dumay et al. (1994) and Van Camp et al. (1997) suggested that HHP-induced aggregation of β-Lg may be partially reversible on subsequent storage.

In HHP treated whole milk, some α-La and β-Lg are also found associated with the milk fat globule membrane (Ye et al., 2004).

The mechanism for high pressure induced denaturation of α-La and β-Lg in milk as well as in whey might be as follows (Huppertz, 2006):

β-Lg unfolds under high pressure, which results in the exposure of the free sulphydryl group in β-Lg. This free sulphydryl-group can interact with other milk proteins (κ-casein, α-La or β-Lg, and perhaps αs_2-casein), through sulphydryl-disulphide interchange reactions. On release of pressure, unfolded α-La and β-Lg molecules, that have not interacted with another protein, may refold to a state closely related to that of native form of these proteins. The close structural similarity of monomeric untreated, and HHP treated β-Lg indicates that the sulphydryl-disulphide interchange reactions occur *during* HHP treatment, since the free sulphydryl-group of β-Lg is not available for interaction after high pressure treatment.

β-Lg exists in several isoforms. Isoforms A and B are the most abundant ones. Pressure stability of these variants were compared by Botelho et al (2000). Pressure denaturation experiments revealed different stabilities of the two isoforms. β-Lg B had higher pressure sensitivity than β-Lg A. It was proposed by the authors that the existence of of a core cavity in β-Lg B may explain its higher pressure sensitivity compared to β-Lg A.

4.3.5.2 Effect of High Hydrostatic Pressure on Caseins

Casein micelles are influenced considerably by HHP treatment. In one of the first studies Schmidt and Buchheim (1970) used electronmicroscopy to examine the size of casein micelles after HHP treatment. Since then several methods have been used to detect changes in casein micelles during or following pressurization, such as transmission electron microscopy, laser granulometry, photon correlation spectroscopy, and turbidimetry.

Casein micelle size is affected only slightly by HHP treatment at pressures below or at 200 MPa at 20°C. HHP treatment at 250 MPa increases average micelle size by ~30% and pressures higher than 300 MPa reduce micelle size by ~50% (Desobry- Banon et al., 1994; Gaucheron et

al., 1997; Needs et al., 2000b; Huppertz et al., 2004b; Huppertz et al., 2004c). Increase in the average size of casein micelles after treatment at 250 MPa is reversible during storage. Increased storage time and temperature enhance the reversibility (Huppertz et al., 2004b). Pressurization at 400 MPa or at 600 MPa broke up all large micelles into smaller fragments (Needs et al., 2000b). Any decreases in micellar size after treatment at higher pressure (300-800 MPa) are irreversible during storage.

Fragmentation of casein micelles under pressure is caused partly by the solubilisation of colloidal calcium phosphate, and partly by the dissociation of hydrophobic and electrostatic interactions (Schrader, Buchheim, 1998; Needs et al., 2000b). Micellar calcium phosphate (MCP) is believed to play an important role in maintaining the integrity of casein micelles. The framework of the casein micelles is formed by so-called nanoclusters, that consist of an amorphous MCP core, which is surrounded by a multilayer of caseins. Solubilisation of MCP leads to the disruption of calcium phosphate nanoclusters, and thus weakens the integrity of the micelles. HHP readily disrupts electrostatic interactions that further promote micellar disruption. Micellar caseins may re-associate under prolonged pressurization at 200-300 MPa, because hydrophobic bonds are favoured over hydrophobic solvation. Re-association doesn't take place at higher pressure (Huppertz et al., 2006). Upon increasing the calcium concentration in a calcium caseinate suspension, micelles become more resistant to pressure-induced disruption (Lee et al., 1996; Anema et al., 1997). Introduction of calcium to the system most likely shift the calcium equilibrium from the soluble to the colloidal phase.

HHP treatment increases the hydration of casein micelles. This is partly due to the association of denatured β-Lg with the casein micelles. Thus, the net negative charge on the micelle surface increases and enhances micellar solvation. HHP induced disruption of micelles further increases micellar hydration, which increases with decreasing micelle size, and is higher for irregularly-shaped than spherical particles (Huppertz et al., 2006).

High hydrostatic pressure (100-400 MPa) significantly increased the transfer of individual caseins from the colloidal to the soluble phase of milk from several species (López-Fandiño et al., 1998). The order of the dissociation of casein variants in bovine milk was as follows: $\beta>\kappa>\alpha_{s1}>\alpha_{s2}$. In goat's, and ewe's milk the order was different: $\kappa>\beta>\alpha_{s2}>\alpha_{s1}$ casein (Huppertz et al., 2002).

Temperature affects micelle size of HHP treated milk. For example when reconstituted skim milk was pressurized at 250 MPa, 20°C, HHP treatment didn't cause significant effect on micelle size. When HHP treatment was carried out at 40°C, micelle size increased, at 4°C micelle size decreased (Gaucheron et al., 1997).

Whether milk received some kind of heat treatment before HHP treatment or not, also influences the effects of pressure on casein micelles. In ultra-high temperature (UHT)-treated skim milk HHP treatment (100–500 MPa) reduced its turbidity, but to a lesser extent than in raw or pasteurised skim milk. This suggests that casein micelles in raw milk, or milk samples heated to lower temperature, are more sensitive to pressure than casein-whey protein complexes that are formed in UHT-treated milk (Buchheim et al., 1996a; Schrader, Buchheim, 1998).

Casein dissociation in milk under pressure (400 MPa) is affected by pH, too. Relative increase in the amount of soluble caseins in milk with pH adjusted to 5,5 or 7,0, was higher than in milk at pH 6,7 (Arias et al., 2000).

4.4 Polyacrylamide Gel Electrophoresis

Electrophoresis is a separation technique that is based on the the mobility of ions in an electric field. Positively charged ions migrate towards a negative electrode and negatively-charged ions migrate toward a positive electrode. Ions have different migration rates depending on their total charge, size, and shape, and can therefore be separated (Tissue, 1996).

Powerful electrophoretic techniques have been developed to separate macromolecules on the basis of molecular weight. The mobility of a molecule in an electric field is inversely proportional to molecular friction which is the result of its molecular size and shape, and directly proportional to the voltage and the charge of the molecule. Proteins can be resolved electrophoretically in a semi-solid matrix strictly on the basis of molecular weight if, at a set voltage, these molecules are charged to the same degree and to the same sign. Under these conditions, the mobility of the molecules is inversely proportional to their size.

This idea is exploited in PAGE to separate polypeptides according to their molecular weights. In polyacrylamide gel electrophoresis (PAGE), proteins charged negatively by the binding of the anionic detergent sodium dodecyl sulfate (SDS) separate within a matrix of polyacrylamide gel in an electric field according to their molecular weights.

Polyacrylamide is formed by the polymerization of the monomer molecule-acrylamide crosslinked by N,N'-methylene-bis-acrylamide (BIS). Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (-N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves nor when mixed together.

The advantage of acrylamide gel systems is that the initial concentrations of acrylamide and BIS control the hardness and degree of crosslinking of the gel. The hardness of a gel in turn controls the friction that macromolecules undergo as they move through the gel in an electric

field, and therefore affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix.

4.4.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS or sodium lauryl sulfate) is an anionic detergent which denatures protein molecules without breaking peptide bonds. It binds strongly to all proteins and creates a very high and constant charge:mass ratio for all denatured proteins. After treatment with SDS, irrespective of their native charges, all proteins acquire a high negative charge.

Denaturation of proteins is performed by heating them in a buffer containing a soluble thiol reducing agent (e.g. 2-mercaptoethanol; dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues to free sulfhydryl groups, and heating in SDS disrupts all intra- and intermolecular protein interactions. This treatment yields individual polypeptide chains which carry an excess negative charge induced by the binding of the detergent, and an identical charge:mass ratio. Thereafter, the denatured proteins can be resolved electrophoretically strictly on the basis of size in a buffered polyacrylamide gel which contains SDS and thiol reducing agents.

SDS-PAGE gel systems are useful in analyzing and resolving complex protein mixtures. In addition, the mobility of polypeptides in SDS-PAGE gel systems is proportional to the inverse of the log of their molecular weights. This property makes it possible to measure the molecular weight of an unknown protein with an accuracy of +/- 5%, quickly, cheaply and reproducibly (Schmieg, 2004).

4.4.2 Discontinuous SDS Polyacrylamide Gel Electrophoresis

Disc gels are constructed with two different acrylamide gels, one on top of the other. The upper or stacking gel is a very loose gel, while the lower resolving gel (or the running gel), contains a higher acrylamide concentration, or a gradient of acrylamide.

Both gels can be cast as thin slabs between glass plates, an arrangement which improves resolution considerably, and which makes it possible to analyze and compare many protein samples at once, and on the same gel (slab gels).

The goal of these gels is to maximize resolution of protein molecules by reducing and concentrating the sample to an ultrathin zone (1-100 nm) at the stacking gel/running gel boundary. The protein sample is applied in a well within the stacking gel and then overlaid with a running buffer. The arrangement is such that the top and bottom of the gel are in running buffer to make a closed circuit.

As current is applied, the proteins start to migrate downward through the stacking gel toward the positive pole, since they are negatively charged by the bound SDS. Since the stacking gel is very loose, low and average molecular weight proteins are not impeded in their migration and move much more quickly than in the running gel.

The rapid migration of proteins through the stacking gel causes them to accumulate and stack as a very thin zone at the stacking gel/running gel boundary, and the stack is arranged in order of mobility of the proteins in the mixture. This stacking effect results in superior resolution within the running gel, where polypeptides enter and migrate much more slowly, according to their size and shape.

When the most mobile proteins reached the bottom of the gel, current is turned off. Gels are removed and stained with a dye, Coomassie Brilliant Blue. Coomassie blue binds strongly to all proteins. Unbound dye is removed by extensive washing of the gel. Blue protein bands can thereafter be located and quantified since the amount of bound dye is proportional to protein content. Stained gels can be dried and preserved, photographed or scanned with a recording densitometer to measure the intensity of the color in each protein band (Schmieg, 2004).

4.4.3 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

Proteins retain their higher-order structure and often retain their biological activity under native polyacrylamide gel electrophoresis conditions. SDS and β-mercaptoethanol (β-ME) are omitted from the SDS-PAGE protocol. In this case many factors, including size, shape, and native charge determine the migration of proteins. Another result of leaving out SDS is that it doesn’t disrupt the secondary, tertiary and quaternary structures of the protein to produce a linear polypeptide chain, so protein aggregates, which could be formed for example during HHP treatment are not disrupted, they remain intact. (Kurien, Scofield, 2005). These aggregates can’t enter into the running gel but remain in the stacking gel. Because of this, changes caused by HHP become visible by native-PAGE. (Hanula-Kövér, 2006). The resolution is generally not as high as that of SDS–PAGE, but the technique is useful when the native structure or enzymatic activity of a protein must be assayed following electrophoresis (Kurien, Scofield, 2005).

4.5 Two-dimensional Polyacrylamide Gel Electrophoresis (2D PAGE)

Two-dimensional electrophoresis is an orthogonal separation technique by means of which proteins are separated through a process based on two different physicochemical principles. Proteins (polypeptides) are first separated on the basis of their (pH-dependent) net charges by isoelectric focusing (IEF), and further separated on the basis of their molecular weights by electrophoresis in the presence of SDS. Both procedures are carried out in polyacrylamide gels. IEF and SDS-PAGE are both high-resolution techniques (Garfin, 2003).

2D PAGE is very useful when separating proteins having very similar molecular weights or isoelectric points, that couldn't have been separated merely by IF or SDS-PAGE (Ong and Pandley, 2001; O'Donnell et al., 2004).

But 2D PAGE has its disadvantages as well. It can not be used for the separation of polypeptide chains with molecular weights higher than ~150 kDa and lower than ~8 kDa. Low amounts of proteins are difficult to detect. Besides strongly alkaline (pI>12) or acidic (pI<3) proteins can be separated only with difficulty (Ong and Pandley, 2001; O'Donnell et al., 2004).

4.5.1 Isoelectric Focusing (IEF)

In the first dimension proteins are separated according to their isoelectric points. Proteins, depending on the pH of their environment can have positive (+), negative (-) or no (0) charge. Isoelectric point is the pH where the proteins are uncharged. Proteins have (+) charge at pH below their pI, and (-) charge at pH above their pI (Garfin and Heerdt, 2002).

In an electric field the negatively charged proteins move toward the anode, the positively charged ones to the cathode in a given pH range and their velocity depends on the magnitude of their net charge. During migration the proteins either pick up or give off protons, while continously loosing their charge. Their velocity is decreasing and finally the proteins stop at the pH equal to their pI (Figure 9.). Strips of filter paper soaked in electrode solution serve the purpose of stabilising the pH gradient (Garfin and Heerdt, 2002).

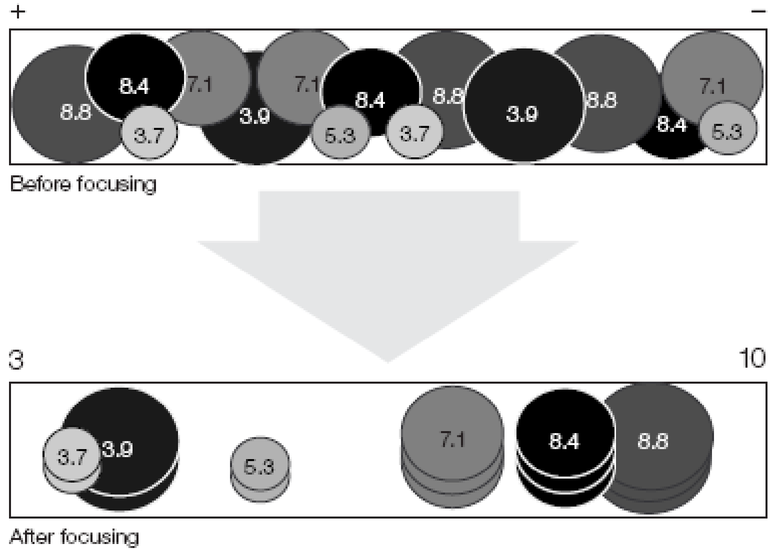


Figure 9. A mixture of proteins is resolved on a pH 3–10 IPG strip according to each protein's pI and independently of its size

The second step in 2D-PAGE is when the proteins, previously separated according to their pI on a strip, are separated according to their molecular weight by SDS-PAGE (as described above) in a direction perpendicular to the direction of IEF.

4.6 Immunoblotting

Immunoblotting (or Western blotting) allows the transfer of proteins from an SDS polyacrylamide gel to an adsorbent (usually nitrocellulose) membrane. Electroblotting is the most commonly used procedure to transfer proteins from a gel to the membrane for example by placing the gel-membrane sandwich between absorbent paper soaked in transfer buffer (semi-dry transfer) for example. The blotted proteins form an exact replica of the gel and are easily accessible to antibodies and special reagents, so detection of proteins by employment of antibody probes directed against the nitrocellulose bound proteins is possible (Fig. 10.) (Kurien, Scofield, 2005).

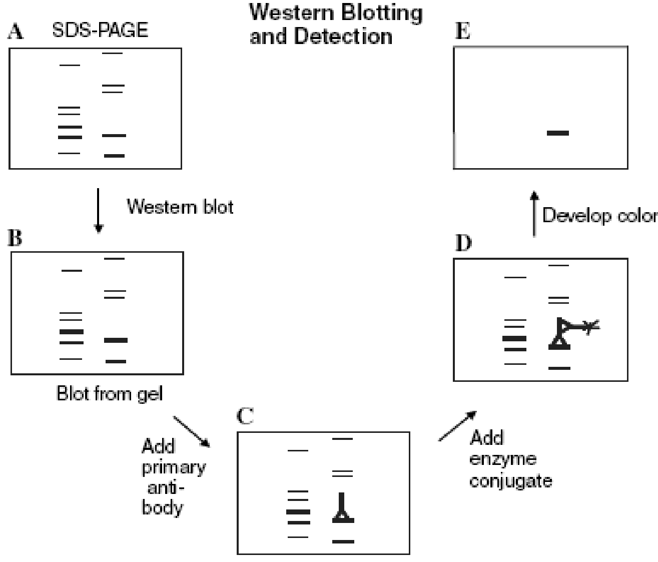


Figure 10. Scheme of immunoblotting and detection (Kurien, Scofield, 2005)

One way of detection is through immunoreaction. First the places on the membrane, where no proteins were bound have to be saturated. As a result antibodies can bind only to the selected proteins and form an antigen-antibody complex. Finally, by the addition of a substrate, insoluble, colourful products appear on the spots corresponding to the proteins with biological activity.

4.7 Fluorescence Spectroscopy

In recent years, the attention and interest of researchers and experts in the field of quality control in the food industry has turned in the last years towards non-destructive, non-invasive, rapid, but at the same time exact and well-reproducible measurement techniques instead of the traditional, often tedious and time-consuming analytical methods. Nowadays even the environmental friendly aspects of a given method are gaining more and more importance (Deshpande, 2001).

For the identification and quantitation of numerous compounds classic biochemical techniques are used in the food research and industry, but fluoreescence-based techniques seem to have been rarely used for this purpose. Although fluorescence was one of the earliest instrumental techniques available to analysis, only recent developments in instrumentation and sample handling have only now made it possible for its full potential to be realised in routine analysis (Deshpande, 2001).

Fluorescence (the name comes from the fluorescent mineral fluorspar) refers to cold light emission (luminescence) by electron transfer in the singlet state when molecules are excited by

photons. Fluorescence is a three-stage process that occurs in certain molecules called fluorophores or fluorescent dyes.

1.) The fluorophore is excited to an electronic singlet state by absorption of an external photon ($h\nu_{ex}$).
2.) The excited state undergoes conformational changes and interacts with the molecular environment in a number of different ways, including vibrational relaxation, quenching, and energy transfer.
3.) A photon ($h\nu_{em}$) is emitted at a longer wavelength, while the fluorophore returns to its ground state.

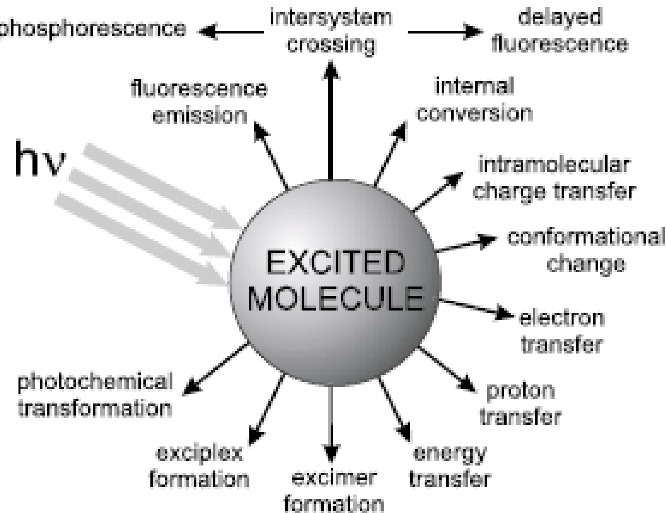


Figure 11. Possible de-excitation pathways of excited molecules (Valeur, 2001).

Once a molecule is excited by absorption of a photon, it can return to the ground state with emission of fluorescence, but many other pathways for de-excitation are also possible (Fig. 11): internal conversion (i.e. direct return to the ground state without emission of fluorescence), intersystem crossing (possibly followed by emission of phosphorescence), intramolecular charge transfer and conformational change (Valeur, 2001). Fluorescent radiation always occurs at wavelengths longer than the exciting wavelength by a wavelength interval depending on the energy loss in the excited state due to vibrational relaxation. This separation between the excitation and emission band maxima is known as Stoke's shift (Deshpande, 2001). The fluorescence *excitation* and *emission* of light typically appears within nanoseconds and is independent of temperature. The molecular structure and environment is decisive for whether a compound is fluorescent. Fluorescence is often exhibited by organic compounds with rigid molecular skeletons, usually polyaromatic hydrocarbons and heterocycles. The less vibrational and motional freedom in the molecule, the greater the possibility that the difference in energy

between the excited singlet state and the ground electronic state is sufficiently large to cause deactivation by fluorescence (Christensen et al., 2006).

Fluorescence is unique among spectroscopic techniques, because it is multidimensional. Two spectra (i.e. excitation and emission spectra) are available for identification of a certain compound, instead of one (e.g. absorption spectrum). The excitation spectrum is obtained by measuring the fluorescence intensity at a fixed emission wavelength, while the excitation wavelength is scanned. For most large, complex molecules, the excitation spectrum is quite stable, and doesn't depend on the emission wavelength at which it is monitored. The emission spectrum is obtained by measuring the fluorescence intensity at a fixed excitation wavelength, while the emission wavelength is scanned. If the shape of the emission spectrum changes with changing wavelengths of the exciting light, the presence of more than one fluorescent compound should be suspected (Deshpande, 2001). Besides the high specificity of fluorescence spectroscopy, the Stokes shift is fundamental to the sensitivity of the fluorescence measurements. Concentrations as low as 10^{-10} to 10^{-12} M can be easily detected.

Food contains a few naturally occurring fluorescent compounds that are important for the nutritive, compositional, and technological quality, such as aromatic amino acids (like tryptophan), vitamins and cofactors, nucleic acids, porphyrins, flavonoids, coumarins, alkaloids, and myco- and aflatoxins (Christensen et al., 2006).

Although fluorescence measurements do not provide detailed structural information, the technique has become quite popular because of its sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes (Royer, 1995).

As a consequence of the strong influence of the surrounding medium on fluorescence emission, fluorescent molecules are currently used as probes for the investigation of physicochemical, biochemical and biological systems. Fluorescent probes can be divided into three classes: (1) intrinsic probes; (2) extrinsic covalently bound probes; and (3) extrinsic associating probes. Intrinsic probes are ideal but there are only a few of them (e.g. tryptophan in proteins). The indole group of Trp is the dominant fluorophore in proteins. Indole absorbs around 280 nm and emits around 340 nm. The emission spectrum of indole may be blue shifted if the group is buried within a native protein, and its emission may shift to longer wavelength (red shift) when the protein is unfolded (Lakowicz, 1999). When an analyte is fluorescent, direct fluorometric detection is possible by means of a spectrofluorometer operating at appropriate excitation and observation wavelengths. This is the case for aromatic hydrocarbons, proteins, some drugs, chlorophylls, etc. (Valeur, 2001).

Experimentally, the efficiency of light absorption at a wavelength λ by an absorbing medium is characterized by the absorbance A (λ) or the transmittance T (λ), defined as:

$$A(\lambda) = \log \frac{I_\lambda^0}{I_\lambda} = -\log T(\lambda)$$
$$T(\lambda) = \frac{I_\lambda}{I_\lambda^0}$$
Equation 3. (Valeur, 2001)

where I^0_λ and I_λ are the light intensities of the beams entering and leaving the absorbing medium, respectively. In many cases, the absorbance of a sample follows the Lambert-Beer Law:

$$A(\lambda) = \log \frac{I_\lambda^0}{I_\lambda} = \varepsilon(\lambda) l c$$
Equation 4. (Valeur, 2001)

where ε (λ) is the molar (decadic) absorption coefficient (commonly expressed in L $mol^{-1}cm^{-1}$), c is the concentration (in mol L^{-1}) of absorbing species and l is the absorption path length (thickness of the absorbing medium) (in cm) (Valeur, 2001).

In several studies of dairy products fluorescence emission spectra of Trp have been investigated as an indicator of the protein structure. Front-face fluorescence emission spectra were correlated to sensory texture and used for discrimination of the cheese type (Dufour et al., 2001). Molecular interactions during milk coagulation were studied by fluorescence detection (Lopez and Dufour, 2001). Several different coagulation systems were studied, and the fluorescence approach plus multivariate data evaluation allowed the investigation of the network structure and molecular interactions. In other studies fluorescence spectroscopy proved to be the best way to provide relevant information on cheese protein structure that was used to discriminate different ripening stages (Kulmyrzaev et al., 2005). Front-face fluorescence spectroscopy was also suggested as a rapid method for screening of process cheese functionality; (Garimelle Purna et al., 2005) in the presented study functionality was represented by meltability as measured by dynamic stress rheometry. Application of classification methods on fluorescence spectra recorded on Emmenthal cheeses (Karoui et al., 2004; Karoui et al., 2005) from different European geographic origins was shown to give correct classification results for approximately 75% of the samples in the 2004 study and around 45% in the 2005 one.

In a few dairy products, retinol fluorescence has been recorded using excitation spectra with emission at 410 nm. The fluorescence signal has been related to phase transition of triglycerides in cheese (Dufour et al., 2000). A combination of retinol fluorescence and tryptophan fluorescence has been applied in several studies of cheese. The common fluorescence signal was found to correlate with the cheese type, as well as with the structure of soft cheese (Herbert et al., 2000). The rheological characteristics of various cheeses (Kulmyrzaev et al., 2005; Karoui et al.,

2003a; Karoui and Dufour, 2003b; Karoui et al., 2003c) and classification of cheese and milk according to origin (Karoui et al., 2004a; Karoui et al., 2005a; Karoui et al., 2005b) were also possible by spectrofluorometry. A combination of fluorescence assigned to tryptophan (emission spectra using excitation wavelength at 295 nm) and retinol (excitation spectra recording emission at 410 nm) was applied in a front-face fluorescence study of milk (Dufour, 1997). Classifications based on principal component analysis (PCA) of the fluorescence spectra clearly separated raw, heated, and homogenized milk samples.

5 OBJECTIVES

Due to the fact that HHP technology was introduced to the food industry only over the last 15-20 years, data about its effects on different raw materials and foodstuffs are scarce. More research needs to be done regarding the influence of HHP on the microflora, components and functional properties of foods.

Easy access to lab-scale HHP equipment at the Department of Refrigeration and Livestock Products Technology provided the opportunity for me to pursue a research project in my field of specialization, dairy science.

1.) The goal of my research was to learn more about the effect of HHP on different types of milk, especially on milk proteins. In this study, milk proteins were investigated not only using the modern methods of proteomics (1D and 2D gel electrophoresis, isoelectric focusing), but using spectrofluorometry as well.

2.) Another very important question arises when new food processing techniques are introduced, namely, whether the new processing method affects the allergenic potential of a foodstuff, since novel foods can be potential allergens. It is necessary to assess the risk of creating or activating hitherto unseen or not bioavailable immunoreactive structures by introducing new food-production and processing technologies. Thus a further objective of my research was to detect the effect of HHP on the immunoreactivity of milk proteins in different milk types.

3.) According to the literature on the subject, fluorescence measurements do not provide detailed structural information, but because of their sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes, spectrofluorometry can be used well in protein investigations. My aim was to compare the modern methods with spectrofluorometry to find out whether the more rapid, but at the same time well-reproducible and reliable fluorometric method can provide sufficient information about the changes in milk components, since in certain cases it is enough to know whether a process took place or not, and if yes, to what extent.

6 MATERIALS AND METHODS

6.1 Milk Types and Whey

The following milk types were investigated:

- Bovine milk (skimmed and whole)
- Goat milk
- Ewe's milk
- Mare's milk
- Human milk.

Besides the different milk types bovine whey was investigated as well.

Fresh raw bovine milk and goat milk was purchased from the Imre Fuchs, Valkó, Hungary. The pre-treatment (length and temperature of cold storage prior to purchase) of the raw milk was unknown. Ewe's milk was provided by the sheep farm of the Bakonszegi Awassi Lt., Bakonszeg, Hungary. Mare's milk was obtained from the stud-farm of Airvent Légtechnikai Ltd. in Kecskemét, Hungary. Human milk was put at our disposal by the Department of Nutritional Science of the Central Food Research Institute, Budapest, Hungary. It was received from Marianne Polgár MD., Madarász Children's Hospital, Budapest, Hungary. Whey was collected from a cheese factory, Soma's Trade Ltd., Budapest, Hungary.

Before treatments, milk and whey samples were filled into polyethylene terephthalate (PET) wide mouth bottles, with a capacity of 30ml, (Nalge Nunc International, Rochester, NY, USA) and tightly closed.

6.2 Treatment by High Hydrostatic Pressure

The samples in the bottles were high pressure treated in the pressure range of 200 MPa to 600 MPa for different holding times from 5 minutes to 30 minutes and at 4 °C initial temperature. A "Food Lab"900® high pressure rig, model S-FL-850-9-W (Fig. 12.) manufactured by STANSTED Fluid Power Ltd. (UK) was used in batch mode to perform the high pressure treatment. The equipment has a chamber size of 40 mm diameter x 240 mm length and a high lift loading system with a detachable canister. The processing pressure is attained within approximately 3 minutes and the pressure was built up operating a continuous, pressure ramp. The high pressure unit was equilibrated to 4°C ± 1°C by circulating temperature-adjusted water in the cylinder wall of the pressure vessel. On account of these conditions the increase in temperature initiated by pressure was below of protein denaturising influences. Ethyl alcohol

containing 15% castor oil for lubrication and anticorrosion purposes was used as pressure-transmitting medium. The “holding time – final pressure” matrix of the samples is shown in Table 6.

Figure 12. Food Lab® high pressure food processor; A: External cooling system; B: high lift loading system to chamber; C: rack for pump and intensifier; D: control panel.

Untreated samples of the given material were used for control.

Table 6. Matrix of pressure treatments

Time [min]	**Pressure [MPa]**							
	100	200	300	400	500	600	700	800
5			+	+		+		+
10	+	+	+	+	+	+	+	+
20		+		+		+		
30		+		+		+		
40						+		

Not all of the pressure-time treatments were included in the different investigations.

6.3 Heat Treatment

In the fluorescence measurements heat treated samples were compared to the pressurized ones.

Heated milk and heated whey was produced by using a Neslab EX 110 pilot plant thermostat (2000 Watt, 230 Volt model) (Fig. 13.) from Neslab Instruments Inc., (Newington, N.H., USA). Different temperatures were adjusted within a temperature range of +70°C to +100°C with a pump flow set at 15 litres per minute maximum. Because of the great pump flow the samples were heavily shaken, this gave rise to a turbulent flow and a minimal laminar boundary layer and resulted in a continual heating from the surface to the core. Temperature stability was +/-0.01°C. Within the final heat range the holding time was ranging from 5 minutes to 30 minutes for the various conditions examined. The "holding time – final heat" matrix of samples is shown in Table 7.. Following the heat processing step the samples were cooled in ice slush to a temperature of approx. 4°C.

Figure 13. Neslab EX 110 pilot plant thermostat

Table 7. Matrix of thermal treatments (bovine milk)

	A	B	C	D
1	70°C 5min	80°C 5min	90°C 5min	100°C 5min
2	70°C 10min	80°C 10min	90°C 10min	100°C 10min
3	70°C 15min	80°C 15min	90°C 15min	100°C 15min
4	70°C 20min	80°C 20min	90°C 20min	100°C 20min
5	70°C 25min	80°C 25min	90°C 25min	100°C 25min
6	70°C 30min	80°C 30min	90°C 30min	100°C 30min

Goat milk and bovine whey were heated only for 10, 20, and 30 mins, respectively, at the same temperatures as shown above.

6.4 SDS- and Native PAGE

6.4.1 Sample Preparation

Milk samples were defatted by hexan before the investigations.

In the experiments, where the effect of fat content on milk proteins was examined, whole bovine milk and skimmed bovine milk were used. Skimming was performed by a FT15 type (Armfield Ltd., UK) laboratory disc bowl separator. The fat content of the whole bovine milk samples was 4.37%, and that of the skimmed bovine milk samples 0.21% measured by the rapid fat determination method of Lindner.

6.4.2 Methodology

The components of the separating gel (running gel) (see Appendix 1. and 2.) were measured and mixed then pipetted between the previously assembled glass plates. The gap between the plates was 0.75 mm. When the gel has polymerized the stacking gel (Appendix 3.) was poured on top and the comb was placed in the gel sandwich.

Samples were diluted in the sample solvent (Appendix 4., and 5.). When SDS-PAGE was done, samples were boiled for 5 minutes.

After the stacking gel has polymerized, the comb was removed and a tracking dye was injected into the formed wells. Then samples were filled into the wells by a Hamilton pipette. The amount of samples was 3 μl/well. LMW molecular weight standard (Appendix 8.), or α-casein, β-Lg, or α-La standards were introduced in wells near the samples.

Gel was placed in the buffer chamber, followed by the adding of running gel buffer (Appendix 6.) was added into the chamber. After setting the running voltage to 200 V, the running of the gel was started. Gels were run in a BIO-RAD Mini-protean II. cell apparatus.

When the tracking dye reached the bottom of the gel running was stopped and gels removed. Gels were put into 20% tri-chloro-acetic-acid and gently agitated by a shaker for 20 mins for fixing the proteins. Then gels were washed by PAGE-gelwasher solution (Appendix 9.) then stained by Coomassie Brillant Blue R-250 (Appendix 10.). After proper staining gels were de-stained by 10% acetic-acid (Takács, 2003).

6.5 Gradient Gel

When applying this method the concentration of acrylamide in the running gel is increasing from the top towards the bottom of the gel. This gradually increasing concentration is achieved by using a gradient-mixer. The steps following polymerization of the running gel are the same as

in 6.4.2. Gels with 12-20%, and with 5-20% concentrations were used in these examinations (Oroszi, 2005).

6.6 2D-PAGE

Milk samples were diluted (Appendix 11.). For isoelectric focusing 7 cm long IPG strips of 3-6 pH were used (1 strip/sample). Strips were placed into the focusing vat and the diluted samples were pipetted onto the strips (250 µl sample/strip) and covered by mineral oil to avoid drying out. Then the focusing vat was placed on the Bio-Rad Protean IEF Cell apparatus, proper parameters were set (Appendix 12.) and focusing was started.

At the end of IEF strips were shaken for 10 min in DTT (Appendix 13.) and then for another 10 min in iodo-acetamide (Appendix 14.). In the second dimension (SDS-PAGE) proteins were separated in a 12-20% gradient gel. The thickness of the polyacrylamide gel was 1.5 mm. Gels were prepared as described previously in 6.4., and 6.5. Then the strips were placed in the long well of the gel and the molecular weight standards were pipetted into the small well adjacent to the long one. The wells were covered by 0.5% agarose. After its solidification separation was started. The steps that followed were the same as in point 6.4.

6.7 Electrophoretic Immunoblotting

In the process of electrophoretic immunoblotting the run gel and the nitrocellulose membrane were soaked in cold Towbin buffer (Appendix 15.) for 10 minutes. Then the gel-membrane sandwich was placed between sheets of absorbent paper soaked in transfer buffer and put into the Bio-Rad Trans Blot Semi-Dry Transfer Cell. After blotting the membrane was shaken in a fixing then in a covering buffer (Appendix 17., and 18.) and washed in a washing-incubating buffer (Appendix 16.). After washing the antibody, individual milk-positive human blood sera were added. The membrane stayed in it overnight. After washing, the conjugate, horseradish peroxidase-labeled anti-human IgE was added to the membrane, and shaken for 1.5 hours. The membrane was washed, then incubated in cold phosphate buffered saline (PBS) solution (Appendix 19.). Finally the formed complex was made to be detected by the developing substrate (Appendix 20.).

6.8 Evaluation of Electrophoretograms

For evaluation, the gels were scanned with a Bio-Rad Gel Doc 2000 video densitometer using the Quantity One version 4.6.1. software. The densitometer measures the optical density (OD) of the given protein fraction bands after staining. Since the amount of bound dye, Coomassie Brillant Blue, is proportional to the protein content, changes in the amount of protein fractions can be detected. On the densitogram the X axis is the relative front (Rf), i.e. the relative position of the protein bands on the gel, and the Y axis shows the optical density.

6.9 Fluorescence Spectroscopy

6.9.1 Instruments and Principal Functions

Fluorescence spectra of milk and whey samples were obtained using a FluoroMax-3® (Fig. 14.) spectrofluorometer (Jobin Yvon HORIBA, Spex® Instruments Inc., USA), equipped with a single-position (90°) cell holder for fluorescence detection. FluoroMax-3® is a fully automated spectrofluorometer, with a wavelength range of 250 nm and 850 nm and under the control of DataMax spectroscopy software for Windows'98® and Windows'2000®. After the preliminary measurements the single-position cell holder was replaced by MicroMax 384 microwell-plate reader (Fig. 15.). The MicroMax 384 is able to accept plates with up to 384 wells, and can be connected to FluoroMax or to an other compatible spectrofluorometer.

Figure 14. Fluoromax-3 and MicroMax 384

MicroMax 384's high speed allows scanning a complete 96-microwell plate in less than one minute. By moving the microwell plate through stationary optics, the MicroMax 384 ensures high sensitivity, excellent accuracy, and high reproducibility. The typical sensitivity lies at about 10nM fluorescein. Light from the excitation and emission monochromators is carried via a fibre-optic bundle to and from the MicroMax 384, thus it is possible to scan with the main

spectrofluorometer and select any excitation and emission wavelength pair for intensity measurements. All control of the MicroMax 384 is automated through DataMax software; custom selection of microwells on the plate is possible through the software.

A 96 well plate, (Jobin Yvon HORIBA), was used in the measurements with a typical volume of 200 µL for each sample.

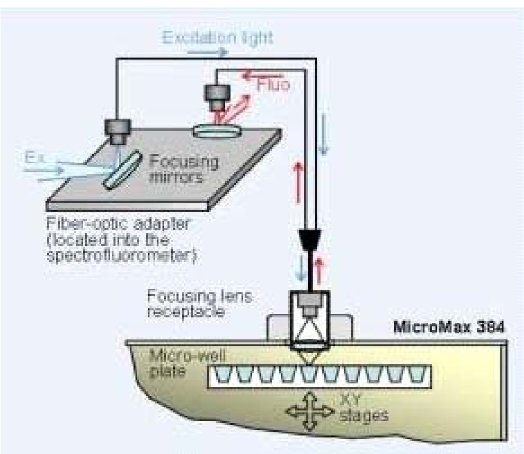


Figure 15. Principle of the MicroMax 348 microwell plate reader (Jobin Yvon HORIBA, 2006).

6.9.2 Calibration

Upon installation and as a part of routine maintenance checks, the examination of the performance of the FluoroMax-3® was done as routine check of the system calibration before each day of use. Scans of the xenon-lamp output and the Raman-scatter band of water were sufficient to verify the system calibration, repeatability and throughput. Calibration was performed as described in the FluoroMax®-3 and MicroMax® 384 Users and Operation Manual (Jobin Yvon HORIBA, 2001). In the xenon lamp test (Fig. 16.) the maximum of the excitation acquisition of the xenon lamp should be at 467,0 + 0.5 nm to guarantee that the results of the experiment will be correct. The maximum of the emission acquisition of the water Raman scan (Fig. 17.) should be at 397,0 + 0.5 nm to guarantee correct results.

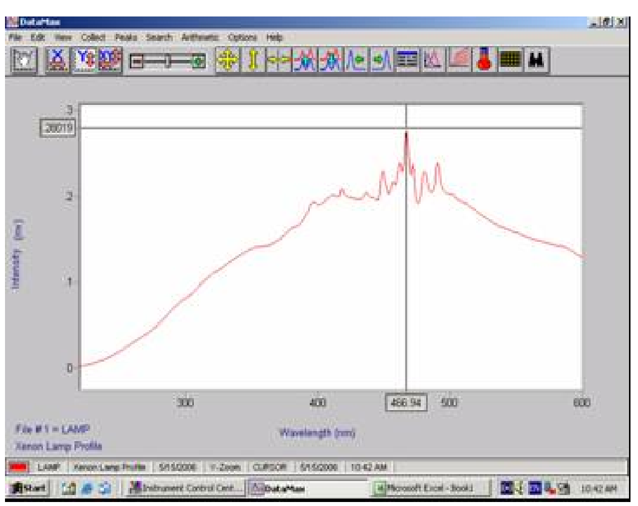


Figure 16. Xenon Lamp Test

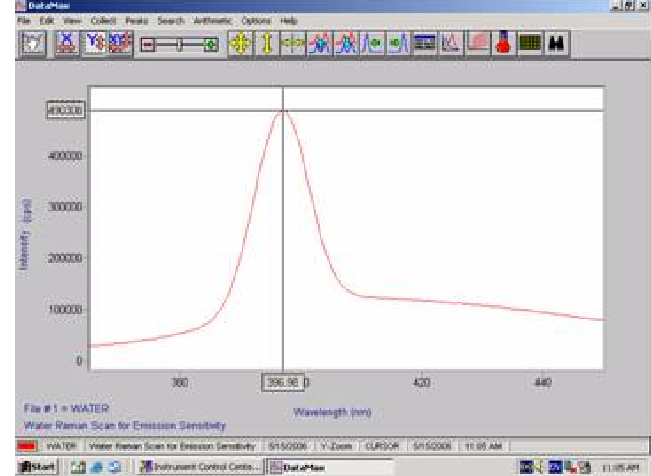


Figure 17. Water Raman Scan for Emission Sensitivity

6.9.3 Software

The DATAMAX® software allows the instrument operation to obtain excitation and emission spectra, total luminescence spectra and time trace. The data processing is done with the same software. Additional capabilities of this program are: the plotting of total fluorescence spectra as isometric projections, three dimensional project maps, contour maps, or level curves where the excitation and emission wavelengths are referenced to the x- and y-axis, and the intensity signals are represented by the z-axis. Furthermore, the program can do auto scaling, correction of small scattering effects and processing of spectra by means of mathematical operations, derived or smoothed.

6.9.4 Settings for Recording the Fluorescence Spectra

If absorbance is less than 0.1, the intensity of the emitted light is proportional to fluorophore concentration. When the absorbance of the sample exceeds 0.1, emission and excitation spectra are both decreased and excitation spectra are distorted. To avoid these problems, a dilution of samples is necessary so a total absorbance of less than 0.1 (Karoui et al., 2003).

The effect of dilution by distilled water on fluorescence intensity of the milk and whey samples was studied by varying the parameter between 1:2 and 1:25. This was done to avoid scattering effects, diffuse reflectance and banked intensity. The analysis of the intensity and the definition of the peaks showed a dilution of about 1:20 as the best for the detection of the emission spectra of tryptophan.

It was found that for the detection of the emission and excitation spectra of retinol, turned out, that a dilution of the samples causes a loss in the fluorescence intensity and an overlapping of two or more characteristic bands. So retinol was measured in an undiluted form.

In the fluorescence measurements the emission spectra of tryptophan, and emission and excitation spectra of retinol were detected in whole bovine milk, goat milk and bovine whey. The parameters of the measurements were as follows (Table 8.) (Strixner, 2006):

Table 8. Settings for the Tryptophan Emission Acquisition

Scan Start [nm] 305.000	Scan End [nm] 450.000	
Increment [nm] 0.500 Excitation [nm] 290.000	Integration [s] 0.1000	
Signals (S) [cps]		
Scan numbers [3]		
Slits [nm] bandpass		
	Excitation 1	5.000
	Emission 1	5.000
Emission Acquisition		
Start time	[Immediate]	

During the scanning of emission spectra of retinol, the intensity of the emitted light was detected within the wavelength range of 350-500 nm, and the wavelength of excitation was 321 nm. The other settings remained the same as shown in Table 8.

When the excitation spectra of retinol were scanned, the emission wavelength was set to 410 nm, and the excitation wavelengths were detected between 380 and 600 nm. Other parameters were unchanged.

In the graphs showing the emission spectra, X axis represents the wavelength in nanometers [nm], and the Y axis the emission intensity in counts per seconds [cps]. This means the number of emitted photons detected on the sensor of the spectrofluorometer in 1 second.

Polynomial fitting was applied on the measurement points using the least squares method (degrees of polynoms ranged from 50 to 200). Emission, and excitation maxima were the local resp. global maxima of these polynoms. Mathematica (Computer Algebra System) was used in the computations.

Mathematical statistical evaluation of the results of the fluorescence measurements was carried out by paired t-test. Given two paired sets X_i and Y_i of **n** measured values, the paired t-test determines whether they differ from each other in a significant way under the assumptions that the paired differences are independent and identically normally distributed.

To apply the test, let

$$\hat{X}_i = (X_i - \overline{X}) \qquad \text{Equation 5.}$$

$$\hat{Y}_i = (Y_i - \overline{Y}), \qquad \text{Equation 6.}$$

then define t by

$$t = \left(\overline{X} - \overline{Y}\right) \sqrt{\frac{n\,(n-1)}{\sum_{i=1}^{n} \left(\hat{X}_i - \hat{Y}_i\right)^2}} \; . \qquad \text{Equation 7.}$$

This statistic has n-1 degrees of freedom.

A table of Student's t-distribution confidence intervals can be used to determine the significance level at which two distributions differ.

7 RESULTS AND DISCUSSION

7.1 Comparision of Protein Composition of Different Milk Types by Electrophoretic Methods

7.1.1 Comparision of Protein Composition of Different Milk Types by SDS-PAGE

Figure 18. shows SDS-PAGE in 12-20% gradient gel of human milk and milks originating from different animal species.

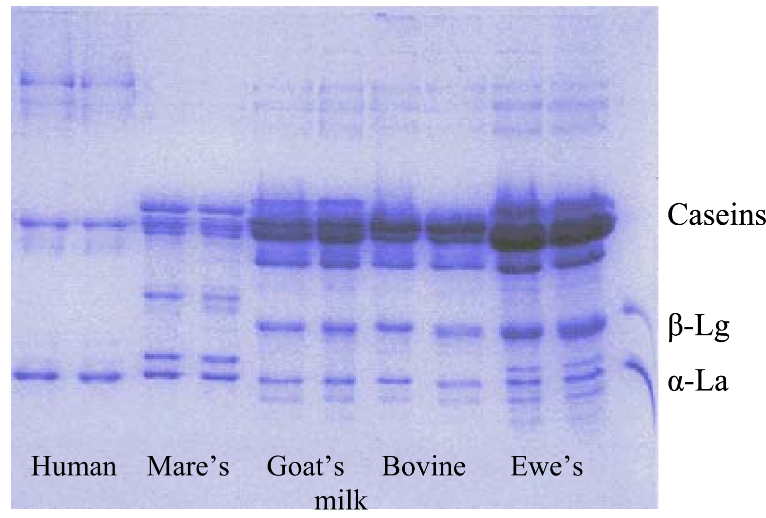


Figure 18. SDS-PAGE of milk samples in 12-20% gardient gel

The gel shows well that human milk and mare's milk, that belong to the albumin milk group, contained much less caseins compared to the other three milk types (Figure 19., see also Table 2.). Caseins appeared in several bands that might indicate the four casein fractions and their genetic variants.

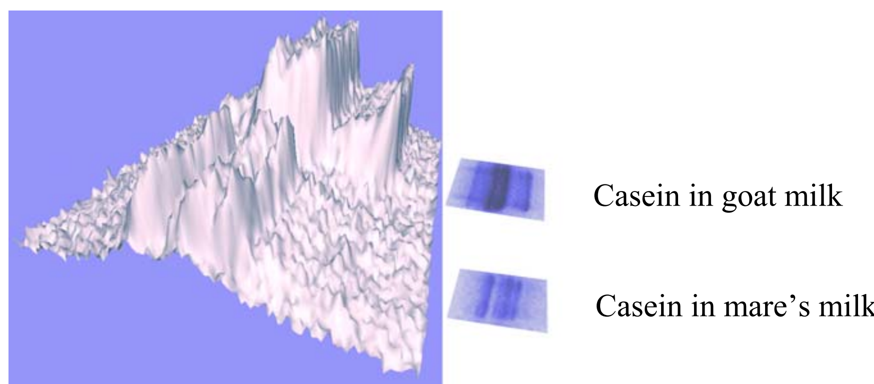


Figure 19. Three-dimensional picture of casein bands in goat milk and mare's milk

As β-Lg is missing from human milk, it is not surprising that no band became visible on the position where this protein fraction is expected. It is interesting that the band of β-Lg of mare's milk was located somewhat higher on the gel than of any other milk investigated (Fig. 20.).

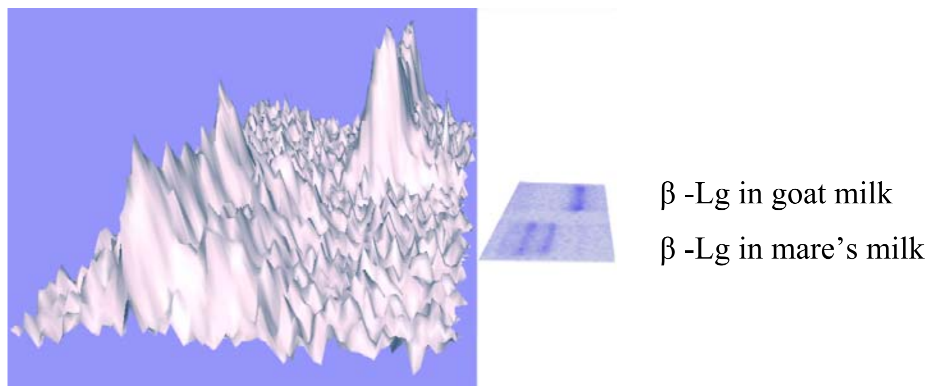


Figure 20. Three dimensional picture of β-Lg bands of goat and mare's milk

Regarding α-La, mare's and ewe's milks were of special interest. In the mare's milk sample one of the two α-La bands that had approximately the same intensity, was located significantly higher compared with human, goat or bovine milk (Figure 21.). In the case of ewe's milk not only two, but four well separated α-La bands appeared on the gel.

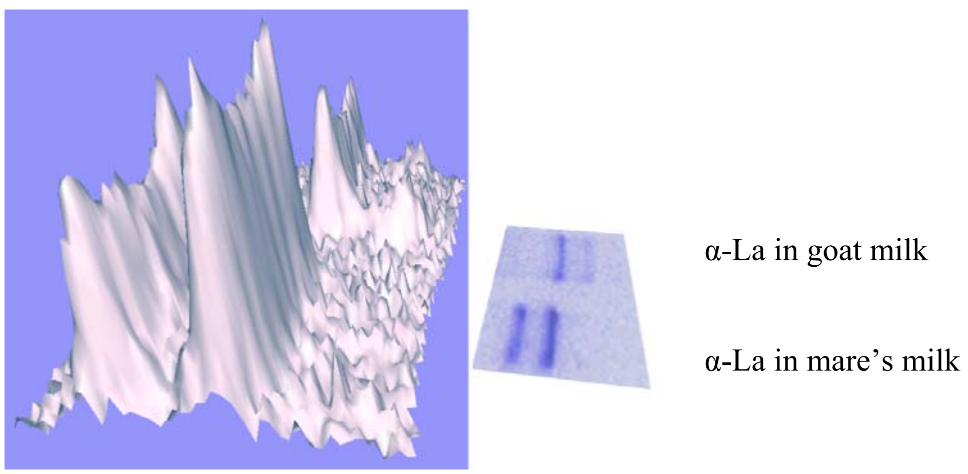


Figure 21. Three-dimensional picture of α-La bands in goat milk and mare's milk

Samples of human, mare’s, goat and bovine milk were examined by 2D-PAGE as well.

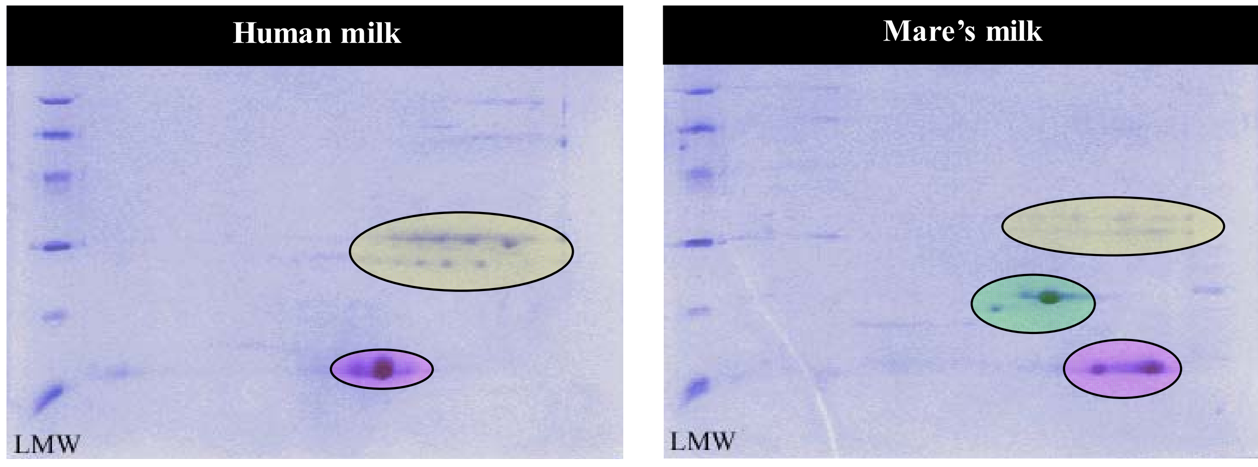


Figure 22/a. 2D-PAGE of untreated human milk

Figure 22/b. 2D-PAGE of raw mare’s milk

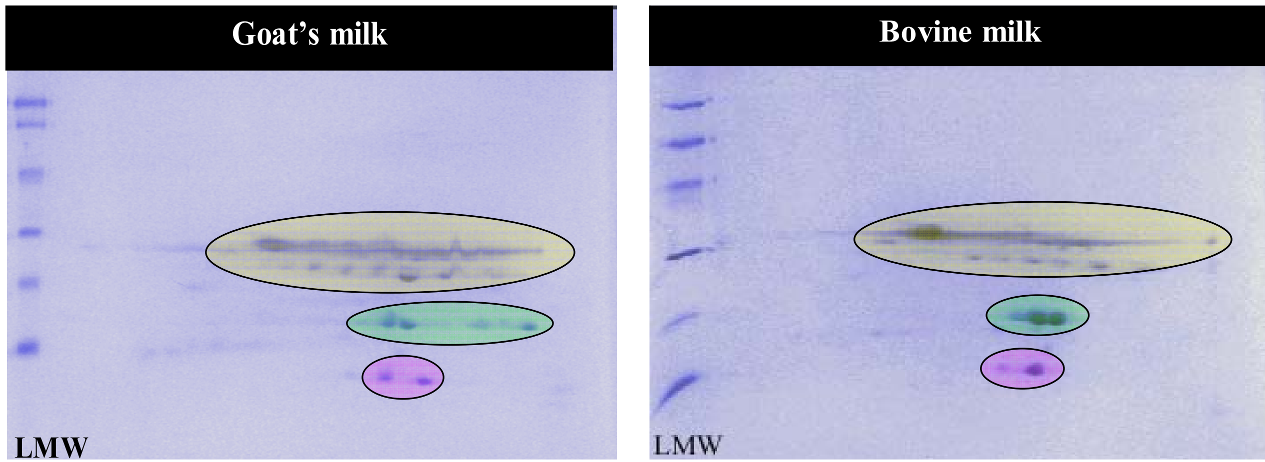
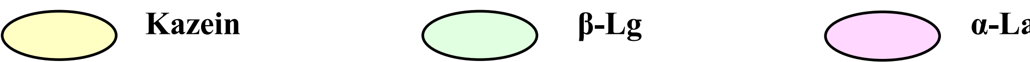


Figure 22/c. 2D-PAGE of raw goat milk

Figure 22/d. 2D-PAGE of raw bovine milk



The series of 2D-PAGE gels in Figure 22. show even more clearly the differences in the amounts of caseins (highlighted by yellow) between the milk samples. Several spots representing this protein appeared for goat milk (Fig. 22/c.) and bovine milk (Fig. 22/d.), and most of them had higher intensities than in the other two milk types. There was no β-Lg (highlighted by green) in human milk. According to these gels, the amount of α-La (highlighted by pink) was less in the goat milk and bovine milk than in human or mare’s milks. Although the number of spots representing α-La was the same (2), their intensities were considerably different.

7.1.2 Effect of High Hydrostatic Pressure on the Composition of Different Milk Types

In the investigations presented in this section, each milk sample was pressurized by 600 MPa for 5 minutes.

Ewe's milk was left out from the examinations, because under the applied pressure-time parameters the milk proteins have already been irreversibly denatured, coagulum was formed, and therefore proper sample preparation couldn't be accomplished. Coagulation took place most likely because ewe's milk had about twice as high protein content as the other ones (Fig. 23.).

Figure 23. Photos of different milk types treated at 600 MPa for 5 mins (Photo: Dalmadi)

Figure 24. shows the control and pressurized milk samples separated by native PAGE.

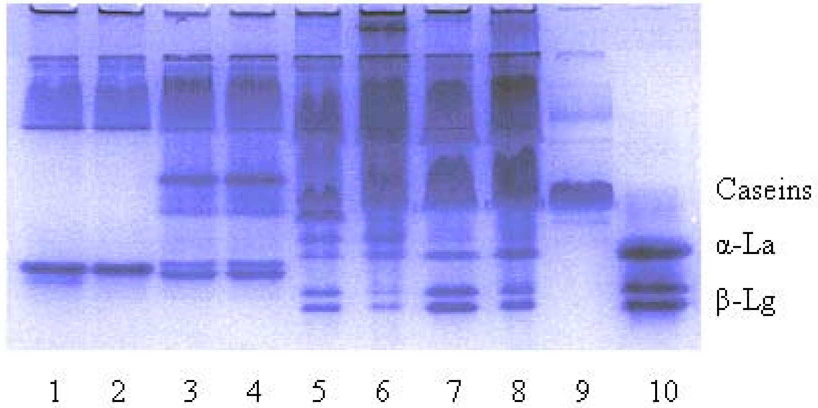


Figure 24. Control and pressurized milk samples separated in 15% native-PAGE

1.Control human milk	2.Pressurized human milk	3. Raw mare's milk	4. Pressurized mare's milk	5. Raw goat milk
6. Pressurized goat milk	7. Raw bovine milk	8. Pressurized bovine milk	9. α-casein standard	10. α-La and β-Lg standards

(On native-PAGE gels α-La and β-Lg appear in opposite order compared to SDS-PAGE.)

7.1.2.1 Effect of HHP on the Proteins in Human Milk

Figure 25. shows the densitogram of HHP treated human milk. As there is no β-Lg in human milk, only the casein and α-La fractions can be seen.

Very slight or no decrease was observed in the intensity of the casein fractions in human milk.

A slight decrease was found as a result of HHP treatment in the protein fraction of human milk located at the position of α-La.

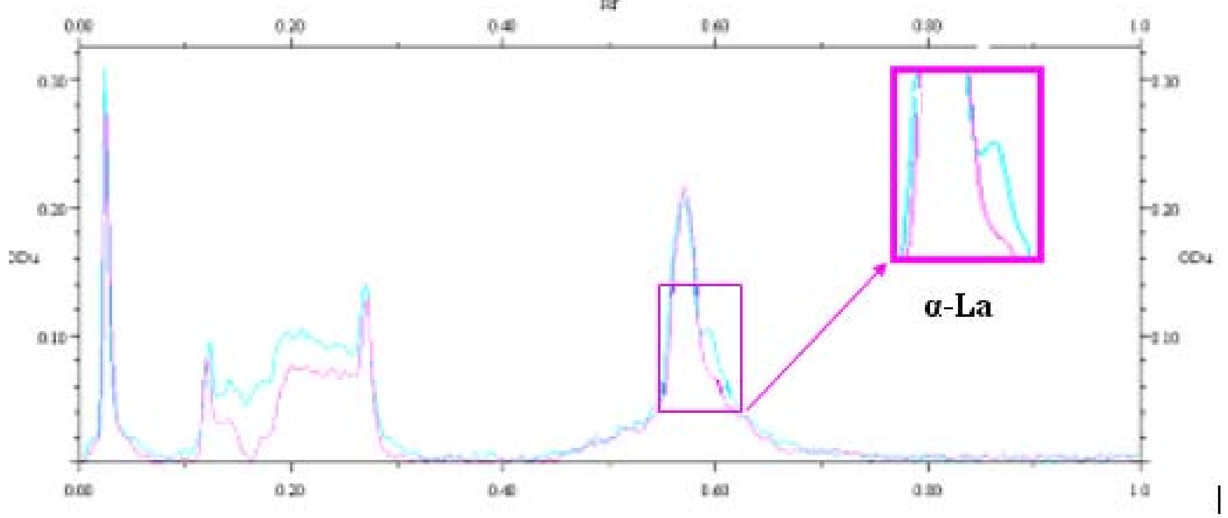


Figure 25. Densitogram of control and pressurized human milk protein fractions separated by native-PAGE.

7.1.2.2 Effect of HHP on the Proteins in Mare's Milk

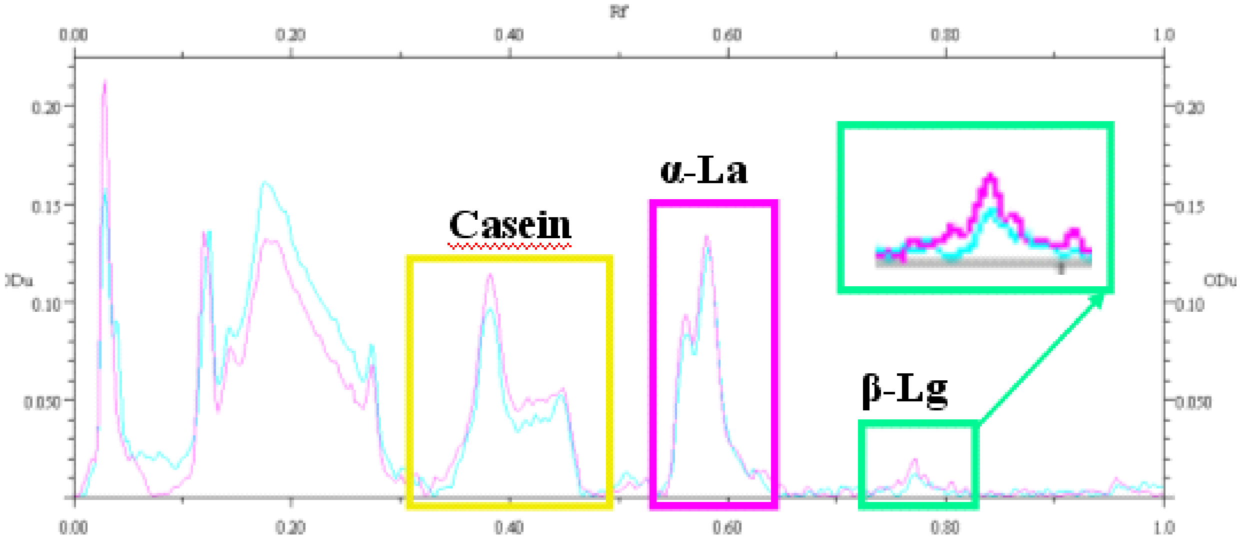


Figure 26. Densitogram of raw and pressurized mare's milk protein fractions separated by native-PAGE

High pressure had a very slight effect on proteins present in mare's milk. The changes could be visualised only on the densitogram (Fig. 26.). Negligible changes occurred in the intensity of the casein fraction. Intensity of the α-La bands increased by about 5%. Intensity of β-Lg increased the most among the other protein fractions, but the increase was not significant.

7.1.2.3 Effect of HHP on Proteins in Goat milk

Protein fractions of goat milk reacted to HHP treatment in different ways (Fig. 27.). Among the two peaks of the protein detected at the position of α-La the first one (lower Rf value) didn’t change, while the second one increased notably, about by 34%. However, the two peaks of the protein fraction, at the position of β-Lg, underwent a significant reduction (~55%) according to the analysis of the densitogram.

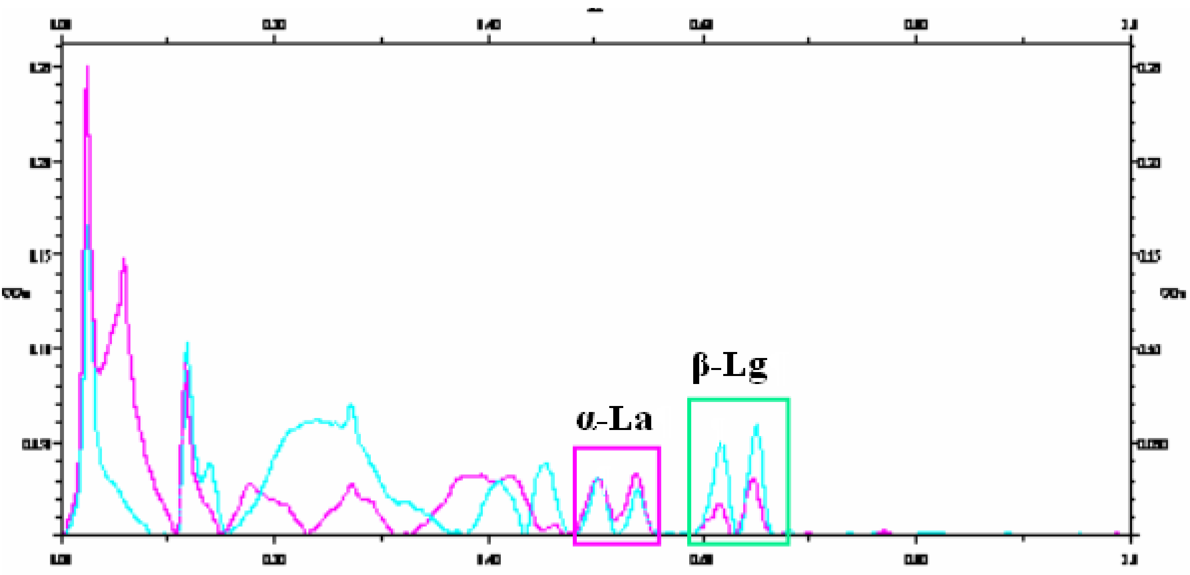


Figure 27. Densitogram of raw and pressurized goat milk protein fractions separated by native-PAGE

7.1.2.4 Effect of HHP on Proteins in Bovine Milk

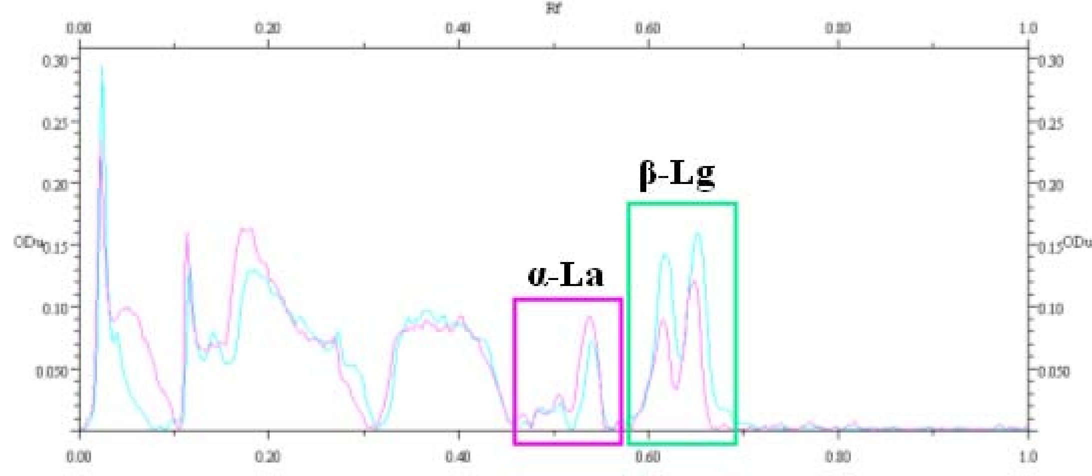


Figure 28. Densitogram of raw and pressurized bovine milk protein fractions separated by native-PAGE

A minimal increase could be observed in the protein fraction detected at the position corresponding to α-La on the densitogram (Fig. 28.). Both bands of the protein fraction, appearing at the position corresponding to β-Lg, changed significantly. The rate of decrease in intensity was ~50%, close to that of goat milk.

7.1.3 More Detailed Investigation of the Effect of HHP on Proteins in Bovine Milk

Since in Hungary bovine milk is consumed in the largest quantity and most dairy products are produced from this type of milk, the effect of HHP on its proteins was investigated in more detail.

7.1.3.1 Effect of the Magnitude of Pressure on Bovine Milk Proteins

The native-PAGE gel (Fig. 29.) shows the changes in protein fractions of bovine milk as a result of increasing pressure. Commercially available pasteurized milk (72°C, 40 s) was also included into the examination. The holding time of HHP treatment was 10 min in each case.

The protein fraction, in which the most apparent changes occurred, was β-Lg. According to the intensity of the bands, β-Lg content of pasteurized milk was approximately the same as the sample's treated by 300 MPa. By increasing pressure β-Lg gradually denatured. The intensity of the bands corresponding to β-Lg was decreasing, and in the sample pressurized to 800 MPa, this fraction was hardly visible. The bands of proteins, having higher molecular weights, showed a more and more diffuse distribution that indicated aggregation. Rademacher at al. (2001) found

that the native β-Lg content decreased at 300 MPa at ambient temperature, and after 60 mins holding time reached ~50% of its original value. Little (~10%) native β-Lg remained after HHP treatment at 800 MPa for 20 min.

In the present separation, no significant changes in α-La and casein content of the different pressurized samples could be observed.

β-Lg appeared on the gels in two bands representing the two isoforms of this protein. The molecular weight and pI of the isoforms is slightly different from each other. Because of their structural differences the two isoforms reacted in another way to pressure. The less mobile isoform denatured first.

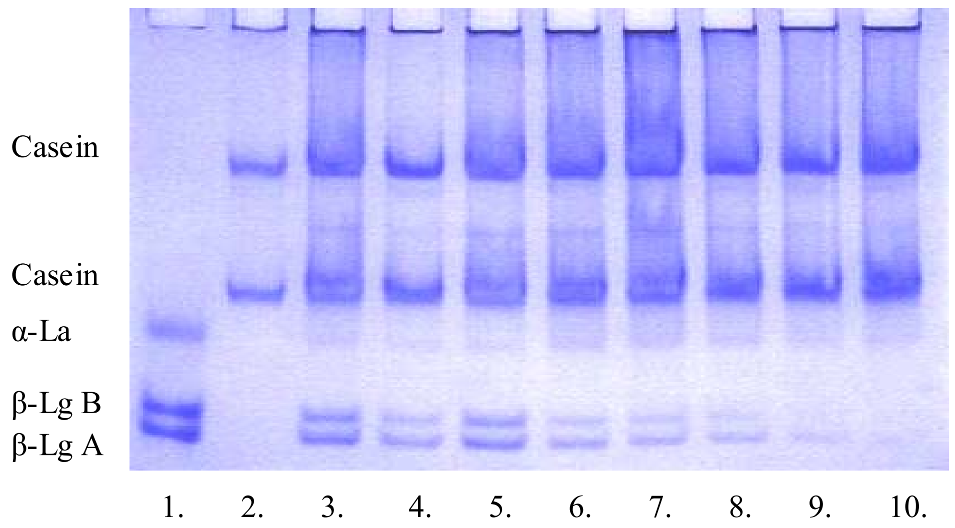


Figure 29. Native-PAGE of bovine milk fractions

1. α-La and β-Lg standards	2. Casein standards	3. Raw bovine milk	4. Pasteurized milk	5.100 MPa, 10 min
6. 300 MPa, 10 min	7. 400 MPa, 10 min	8. 500 MPa, 10 min	9. 700 MPa, 10 min	800 MPa, 10 min

A few samples were investigated in gradient gels as well to achieve more “sharp” separation (Fig. 30.).

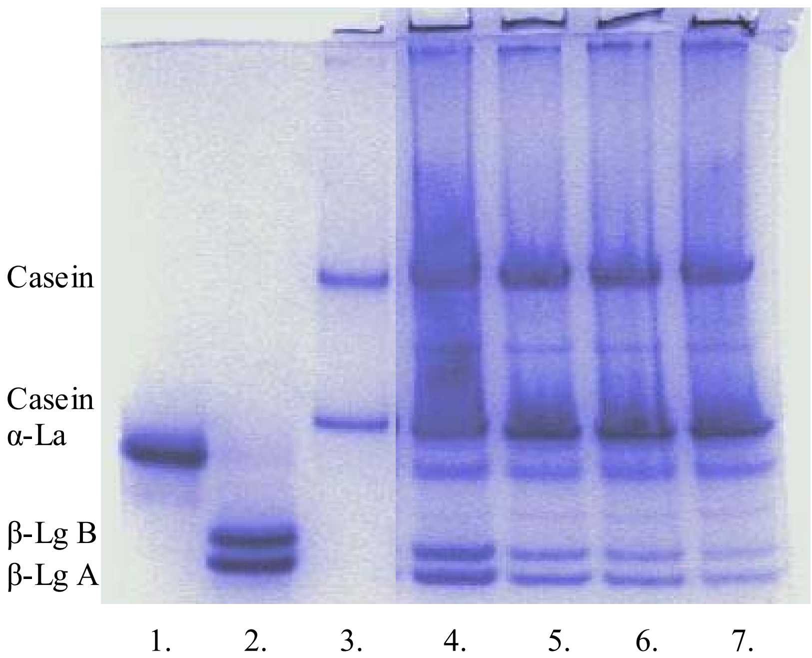


Figure 30. Separation of skim milk samples in gradient gel by native-PAGE

1. α-La standard 2. β-Lg standard 3. Casein standards 4. Control
5. 300 MPa, 5 min 6. 400 MPa, 5 min 7. 600 MPa, 5 min

As an effect of pressure, a new, narrow band appeared between the α-La and β-Lg fractions that wasn’t present or could be only very slightly seen in the control samples.

For the casein standard a pale band could be observed on the top of the running gel. This phenomenon suggests that there have been certain proteins in it, that have entered the running gel but their advance in the gel during running was minimal. The intensity of these bands became stronger when pressure was increased. That means that these proteins might be associates of high molecular weight.

According to the densitogram (not shown), the intensity of the casein bands in pressurized samples increased compared to the control sample, while the intensity of α-La practically hasn’t changed.

Regarding β-Lg, very pronounced changes occurred as an effect of HHP. An enlarged section of the densitogram (Fig. 31.) shows these alterations.

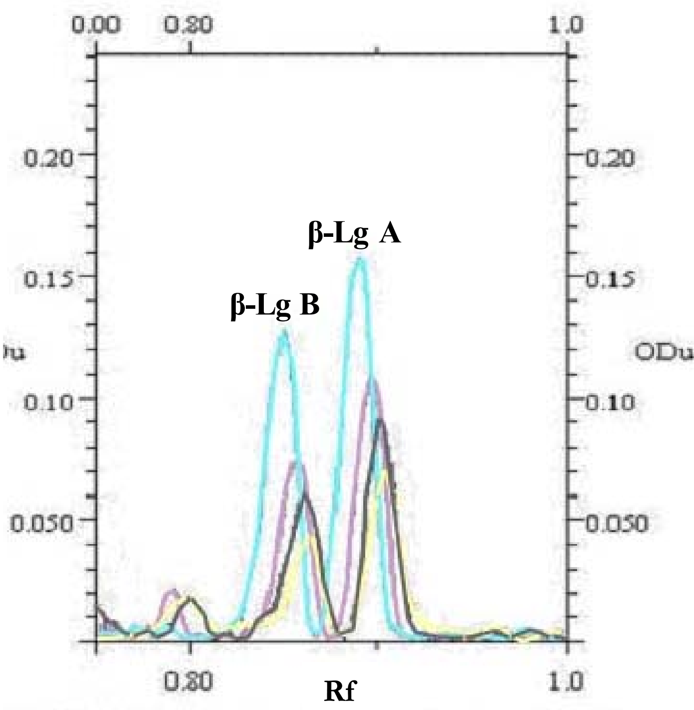


Figure 31. Section of the densitogram showing the optical density of β-Lg bands

Control 300 MPa, 5 min 400 MPa, 5 min 600 MPa, 5 min

The fraction of isoform β-Lg B (lower Rf value) decreased by more than a third of the original value in the sample treated at 300 MPa, and almost to one fourth in the sample treated by 600 MPa. Decrease in the β-Lg A fraction is not as marked as in β-Lg B. Treatment at 300 MPa caused 30% decrease in maximal value of optical density, at 400 MPa a further 12%, and at 600 MPa a total of 57%. The absolute value of the reduction in optical density was very similar in the two fractions, but as the initial amount of β-Lg A was higher, the rate of the reduction proved to be lower than in the isoform B.

7.1.3.2 Effect of Holding Time on Bovine Milk Proteins

Not only the height of pressure but also the holding time influence the food components as well. Fig. 32. presents native-PAGE gel of bovine milk pressurized at constant pressure for different holding times is presented. Although quality of the picture is affected by information loss during digitalisation, it still shows that the longer the holding time, the lower the intensity of the β-Lg bands. Again, β-Lg B proved to be more sensitive to pressure than β-Lg A. Length of holding time didn't seem to have much influence on the intensities of casein and α-La bands based on the present separation.

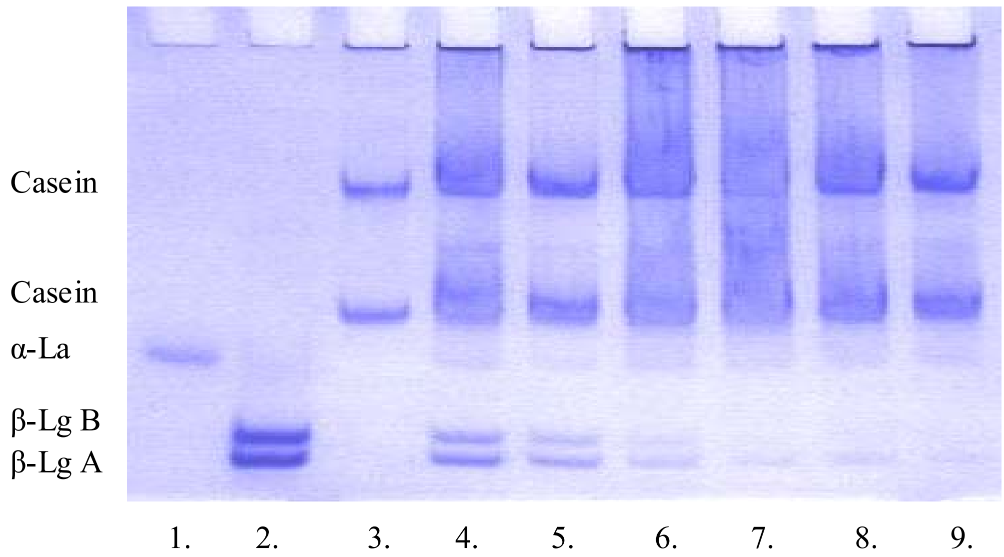


Figure 32. Protein fractions of milk samples treated at 600 MPa for different holding times

1. α-La standard	2. β-Lg standard	3. Casein standard	4. Raw bovine milk	5. Pasteurized bovine milk
6. 600 MPa, 10 min	7. 600 MPa, 20 min	8. 600 MPa, 30 min	9. 600 MPa, 40 min	

7.1.3.3 Effect of Fat Content of Milk on Pressurized Bovine Milk Proteins

Since milk is a complex material, it was expected that the other components, first of all fat, would have an influence on milk proteins on HHP treatment. To examine the interactions between proteins and lipids, we examined the patterns of molecular weight separation of proteins, both in control samples and in pressurized skim and whole milk samples (Fig. 33.).

The fat content of whole milk was 4.37 g/100g, and that of skim milk 0.21 g/100g.

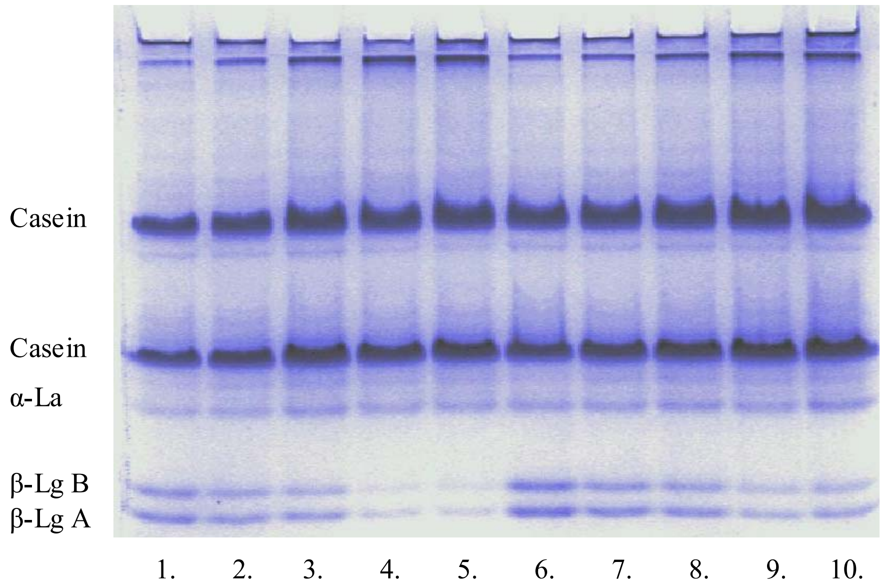


Figure 33. Electrophoretic pattern of whole and skim milk samples by native-PAGE

1. Skim milk control	2. Skim milk, 300 MPa, 5 mins	3. Skim milk, 400 MPa, 5 mins	4. Skim milk, 600 MPa, 5 mins	5. Skim milk, 800 MPa, 5 mins
6. Whole milk control	7. Whole milk, 300 MP, 5 mins	8. Whole milk, 400 MPa, 5 mins	9. Whole milk, 600 MPa, 5 mins	10. Whole milk, 800 MPa, 5 mins

The electrophoretogram demonstrated that the intensity of protein bands changed in a different way in whole and skim milk. Decided differences appeared in the intensities of β-Lg fractions of skim and whole milk samples at 600 and 800 MPa, respectively. The intensity of β-Lg fractions in skim milk decreased more significantly at these pressures than in whole milk.

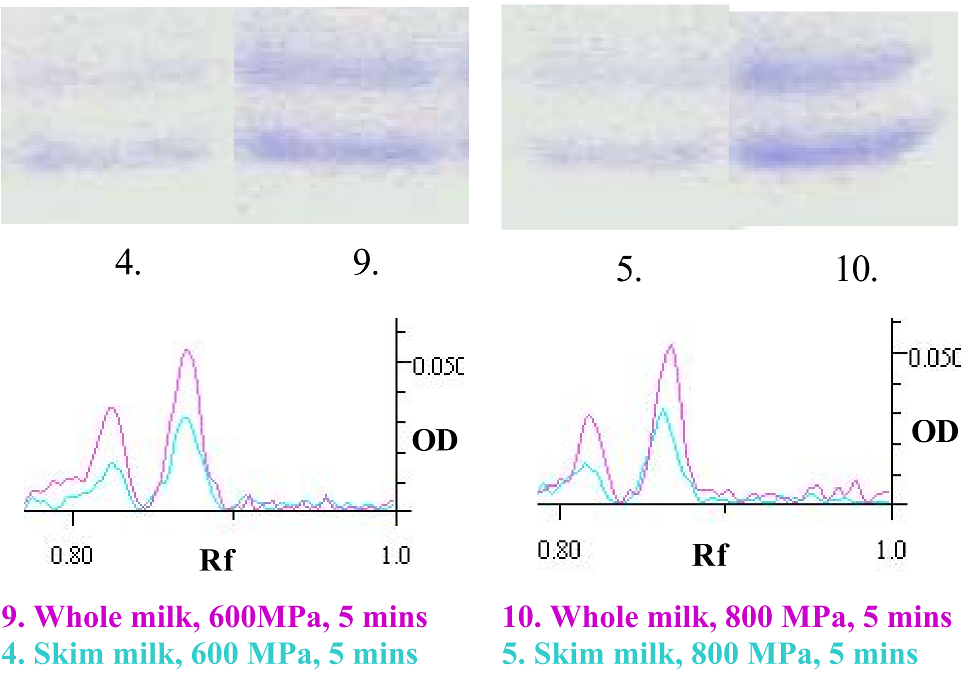


Figure 34. Densitograms of β-Lg fractions in skim milk and in whole milk pressurized at 600 MPa, and 800 MPa, respectively

Enlarging the bands of β-Lg and pairing the skim and whole milk samples treated at the same pressure (Fig. 34.), the contrast is obvious between the milk samples with different fat contents. The densitograms show that ~4% difference in fat content caused about 40% lower intensity of the β-Lg bands of the skim milk sample at the pressures applied. The tests were repeated several times and this phenomenon could be observed each time. This suggests a baroprotective effect of fat on proteins. The literature mentions protective effect of fat against pressure only with regard to the survival of microbes (Gervilla et al., 2000) and to heat denaturation of β-Lg (Pellegrino, 1994). The reason for the very probable baroprotective effect of fat might be the lipid-protein interaction during HHP treatment.

Summarizing the results we found, that intensities of protein fractions in the electrophoretic pattern of HHP treated milk samples decreased with increasing pressure and holding time. The extent of the increase was different in the different milk types, and the milk protein fractions reacted to pressure in a different ways, too.

In the higher pressure ranges, decrease in the intensity of the protein fractions, first of all of β-Lg, was smaller in the whole milk samples, than in skim milk.

Decrease in the amount of detectable proteins can be explained by the (partial) denaturation/aggregation of milk proteins under HHP, and thus their solubility decreased significantly. Whether the non-thermal, mostly reversible denaturation/aggregation of protein fractions was producing advantageous or disadvantageous changes in the conformation and biological activity of milk proteins has yet to be clarified.

7.2 Immunoreactivity of Milk Proteins

Food allergy is an adverse reaction to a food or food component (mainly a protein) involving reactions of the body's immune system. Proteins of several foods have been identified as common allergens, and one of them is milk. Because of its absence in human milk, β-Lg is considered to be one of the major allergenic proteins in cow's milk. Other potent allergens in cow's milk are α_{s1}-casein and Maillard adducts. Goat's and ewe's milk and products made of them show cross-reactivity with sera of patients suffering from bovine milk allergy (Hajós et al., 1997).

Novel foods and novel food ingredients raise the problem of the safety of these foods and require the evaluation of any risks that their consumption could pose to public health. Novel foods appear to be potential allergens. It is necessary to consider the risk of creating or unmasking new immunoreactive structures hitherto unseen or not bioavailable, as a result of new food-production and processing technologies (Wal, 1999).

There are no available data on potential risks of high-pressure processing. However it, is important to clarify the role of HHP with regard to allergenicity and nutritional quality of pressurized foods (Hajós et al., 2004).

The conformational changes of proteins, induced by HHP, may alter antigenicity or immunological cross-reactivity by changing binding abilities of their epitopes (Hajós et al., 2004).

7.2.1 Immunoreactivity of Untreated Milk Samples

The samples of human milk and of different animal species were first separated by SDS-PAGE in 12-20% gradient gel (Fig. 35.) then blotted (Fig. 36.). For immunoblotting milk positive human blood serum was used, and the conjugate was horseradish peroxidase-labeled anti-human IgE.

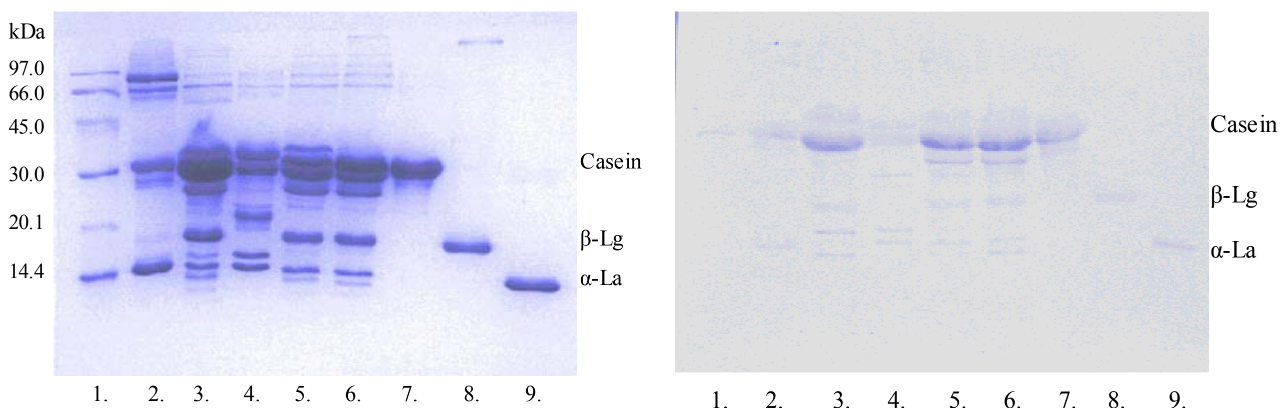


Figure 35. SDS-PAGE of different milk types in gradient gel

Figure 36. Immunoblot of different milk types

1. LMW standard	2. Human milk	3. Ewe's milk	4. Mare's milk	5. Goat's milk
6. Bovine milk	7. α-casein standard	8. β-Lg standard	9.α-La standard	

Immune responses were the strongest in the protein fractions corresponding to casein. Ewe's, goat's and bovine milk gave more intensive responses than the other two milk types. In the goat's and bovine milk samples two immunoreactive bands could be detected.

β-Lg showed immunoreactivity in each milk of animal origin.

The weakest responses were given by human and mare's milk to α-La. In the other three milk types, immunoreactivity caused by this protein fraction could be detected and two active bands were present.

However, when milk positive human serum from an other patient was used in the examinations, the results (not shown) were different. No immune response of α-casein was detected in human and in mare's milk, while the same protein fraction of the other milk types produced a definite immune reaction. Immune responses for β-Lg could be recognised most distinctly in goat's and bovine milk samples, but they were not intensive in either milk sample.

7.2.2 Immunoreactivity of Pressurized Milk Samples

The results of HHP induced changes in the immunoreactivity of milk protein fractions can be best demonstrated on the 2D-PAGE separations.

Control and pressurized (600 MPa, 5 mins) milk samples were first separated by 2D-PAGE in 12-20% gradient gel then blotted. Again, for immunoblotting milk positive human blood serum was used, and the conjugate was horseradish peroxidase-labeled anti-human IgE.

The most promising results were found for mare's and goat milk, and the least changes in immunoreactivity appeared in bovine milk.

Although the 2D-PAGE of control and pressure treated mare's milk samples were very much alike, indicating, that HHP didn't cause any changes in the protein fractions (not shown), the difference between the immunoblots was great. While α-La and β-Lg of control mare's milk gave definite immune responses, no antigen-antibody complex could be detected in pressurized samples (Fig. 37.).

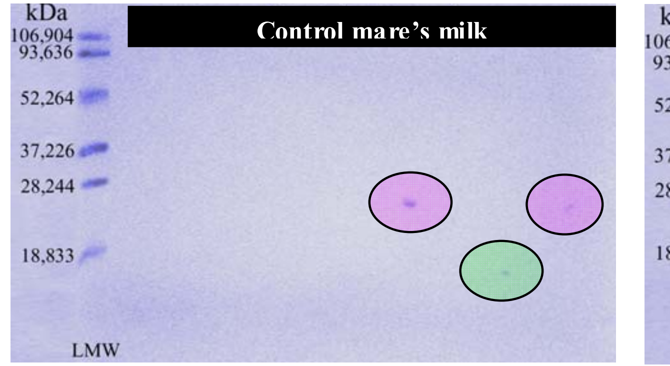


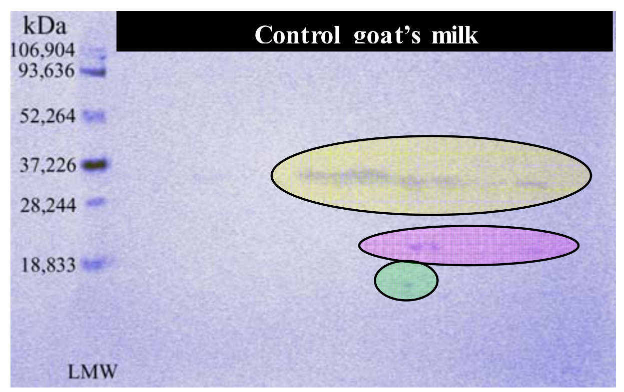
Figure 37. A. Immunoblot of control mare's milk following 2D-PAGE

Figure 37. B. Immunoblot of pressurized mare's milk following 2D-PAGE

β-lg

α-la

2D-PAGE of goat milk didn't show many differences in the intensity of milk protein fractions of control and HHP treated samples (not shown). But again, after immunoblotting, the decrease in the immunoreactivity, primarily in the casein fraction was significant. The intensive line, indicating the casein fraction in control milk, disappeared, only a few spots remained showing immunoreactivity (Fig. 38.). At the same time the intensity of the immune response observed in the position corresponding to β-Lg became slightly weaker. Using the present method, the immunoreactivity of α-La hasn't shown any change.



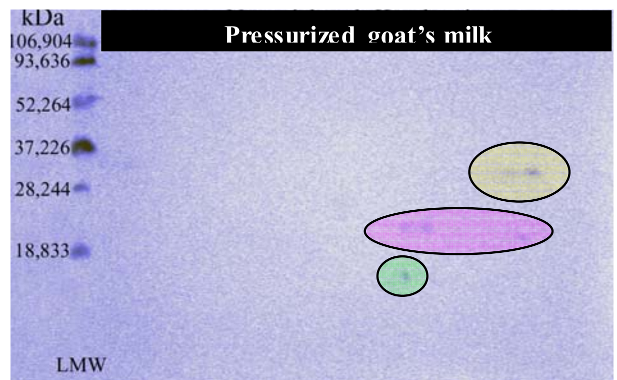
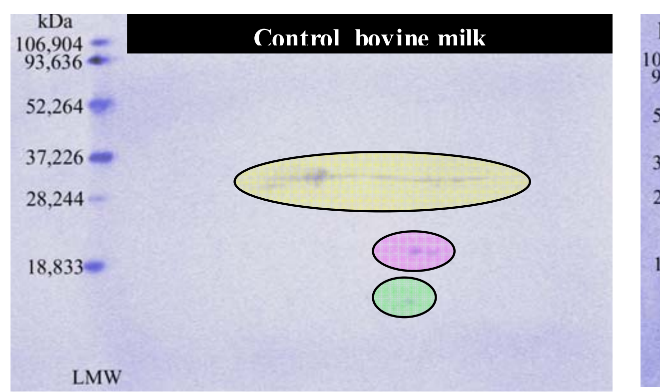


Figure 38. A. Immunoblot of control goat milk following 2D-PAGE

Figure 38. B. Immunoblot of pressurized goat milk following 2D-PAGE

Casein **β-Lg** **α-La**

Changes in the immunoreactivity of protein fractions in bovine milk were found as well. Casein in the pressurized sample gave weaker immune responses than in the control sample. Intensity of the immune reaction caused by β-Lg decreased as a result of pressure treatment. No immunoreactivity of α-La could be found after 5 minutes treatment at 600 MPa (Fig. 39.).



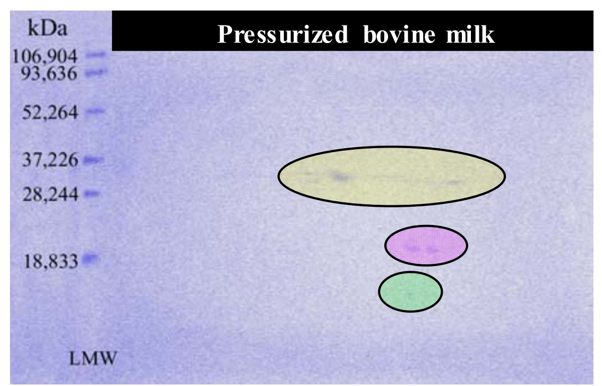


Figure 39. A. Immunoblot of control bovine milk following 2D-PAGE

Figure 39. B. Immunoblot of pressurized bovine milk following 2D-PAGE

Casein **β-Lg** **α-La**

In another test of immunoreactivity of milk proteins, bovine milk was treated applying 300, 400, 600, and 800 MPa each for 5 mins. Antigen-antibody complexes were investigated by using anti-β-lactoglobulin antibody IgG developed in rabbit, and human sera for IgE, respectively.

No differences were detected between the immunoreactivity of casein and α-La fractions neither in control nor in pressurized samples in the measurements with anti-β-lactoglobulin antibody IgG developed in rabbit. But interesting changes occurred in the immunoreactivity of β-Lg. An enlarged section of the densitogram demonstrates it well (Fig. 40.).

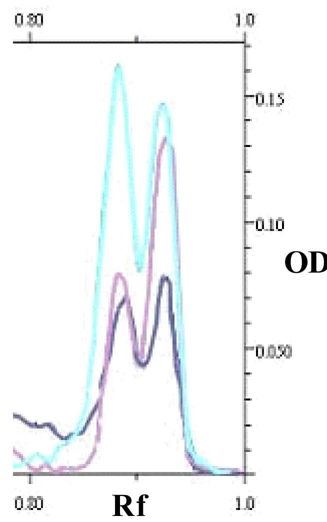


Figure 40. Densitogram section of the immunoblot by anti-β-lactoglobulin antibody IgG developed in rabbit

Skim milk control **Skim milk 300 MPa, 5 min** **Skim milk 600 MPa, 5 min**

The densitogram demonstrates clearly that decrease in immunoreactivity of β-Lg corresponded to the decrease in the intensity of this protein. Three hundred MPa treatment affected β-Lg B (first band from left) in a different way than A (second band from left). At this pressure the intensity of β-Lg B was about half of the original intensity but β-Lg A showed only a very slight decrease. This affirms the finding of Botelho et al. (2000) who reported that β-Lg B was significantly more sensitive to pressure denaturation than β-Lg A. At 600 MPa the intensity of both β-Lg isoforms showed similar values.

Decrease in immunoreactivity could be noticed only in skim milk but not in whole milk according to the applied conditions of the experiment.

When immunochemical reactions with milk positive human serum were studied, casein fractions gave definite responses. High pressure decreased the immunoreactivity of these fractions, but the rate of decrease reached its maximum at 400 MPa treatment. No further reduction was obtained at higher pressures. According to the densitogram (not shown), the ODu value of immunoblotted casein bands changed from 0.060 (control) to 0.037 ODu, an approximate decrease of 40%. The other protein fractions didn't show immunochemical reactions, most likely because the human serum originated from a patient who was sensitive only to casein.

Summing up the results, HHP seemed to decrease the immunoreactivity of certain protein fractions in the different milk types, but the extent of the decrease was not significant, except for mare's milk, according to the applied separation and immunoblotting methods. Thus HHP treatment alone did not prove to be useful in efforts to produce hypoallergenic milk or milk

products. However, in combination with other methods, HHP treatment was effective in decreasing or even cancelling the immunochemical reactivity of milk proteins. Bonomi et al. (2000; 2003) hydrolysed pressurized β-Lg with proteolytic enzymes. The authors found that the immunoreactivity of the whole hydrolysates was related to their content of residual intact β-Lg, and no immunochemical reactivity was found for all the products of chymotrypsin hydrolisis under pressure at 600 MPa. The results indicated that chymotripsin effectively hydrolised hydrophobic regions of β-Lg that had been temporarily exposed during the pressure treatments, and that were not accessible in the native protein or in the protein that had been previously pressure treated.

7.3 Fluorescence Investigations

The attention in the investigations presented in the followings was turned to the alterations caused by high pressure and heat processing in milk proteins by fluorescence spectroscopy.

The materials used in the experiments were whole bovine milk, whole goat milk and bovine whey. The samples were heat treated, and pressurized, respectively. Heat treatment was carried out in a temperature range between +70°C to +100°C increasing the temperature by 10°C steps. Holding times were ranging from 5 mins to 30 mins by 5 min steps. Following heat processing milk samples were immediately cooled in ice-slush to a temperature of 4°C. Parameters of HHP treatment were 200, 400, and 600 MPa, with 10, 20, and 30 min holding times.

7.3.1 Changes in Tryptophan Emission

For the detection of Trp emission spectra, samples were excited at 290 nm and the emission spectra were recorded between 305 nm and 450 nm.

7.3.1.1 Effect of High Pressure or Heat Treatment on Tryptophan Emission Spectra of Whey

Fig. 41. shows the Trp emission spectra of control (untreated) whey samples compared to samples that were pressurized for 30 mins. Intensities of the emission spectral curves were decreasing from 93.000 cps (control) to 79.518 cps (600 MPa) with increasing pressure. This meant a 15% decrease in intensity. The biggest decrease in the intensity of Trp emission was detected between the control samples and samples treated at 200 MPa treated samples. The intensity decreased significantly between samples treated at 200 MPa and at 400 MPa, but its rate was smaller than in the range of 0-200 MPa. Intensities of the Trp emission spectra of the curves representing 400 MPa and 600 MPa treatment were lying close to each other. The big

decrease in intensity between the control samples and those pressurized to 200 MPa pressurized samples was caused presumably by the conformational changes in β-Lg caused by pressure, as it is a barosensitive protein, and its midpoint for transient structural modification during high pressure treatment was reported to be at 150-200 MPa (Dufour et al., 1994; Stapelfeldt et al., 1996). In the pressure range of 200-400 MPa, the conformational changes continued in β-Lg until it was totally denatured by pressure. The smaller decrease in the intensity of tryptophan emission spectra between 400, and 600 MPa hints at conformational changes in α-La, since this protein fraction starts to denature only at pressures higher than 400 MPa (Huppertz et al., 2004), and it is present in bovine milk in a lesser amount (2-5% of total protein in skim milk) than β-Lg (7-12% of total protein in skim milk). When emission spectra of all samples were plotted (not shown), the same tendency was found, namely with increasing treatment conditions the intensity of Trp emission went down step by step from 200 MPa 10 minutes to 600 MPa 30 minutes.

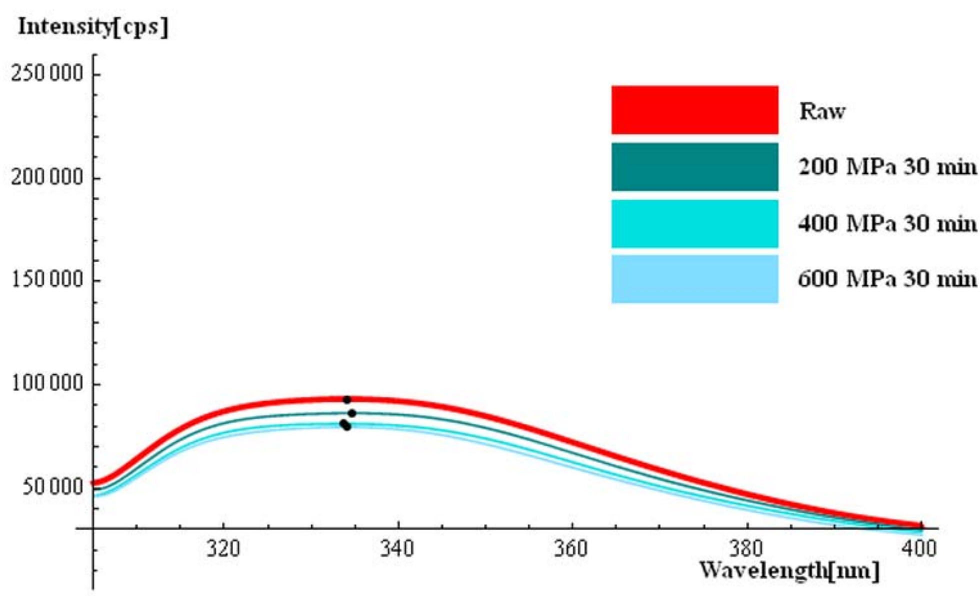


Figure 41. Tryptophan emission spectra of pressure treated bovine whey samples.

Maximum values of emission are marked with black dots on the spectral curves. In case of HHP treated samples, the wavelengths, where the maxima were found, ranged between 333,61 and 334,67 nm. No clear tendency towards red shift or blue shift could be detected. We speak about red shift, when the emission is shifted to a longer wavelength, and about blue shift, when the opposite happens. According to Weber (1987), crystallographic studies have shown that the polarity of Trp environment correlates well with the energy of the fluorescence emission. Weber (1987) observed that the spectral changes in intensity under pressure are accompanied by a shift of the emission to longer wavelengths indicating that at higher pressures the native environment

of the Trp is replaced by one of considerably greater polarity. A simple explanation of this phenomenon is, that at high pressure water molecules penetrate the interior of the protein and they cluster close enough to the Trp residues. This allows strong interaction with the field of the dipole fluorophore.

An opposite tendency was observed in the course of heat treatment of whey (Fig. 42.). Intensity of Trp emission was increasing with increasing temperature and holding time.

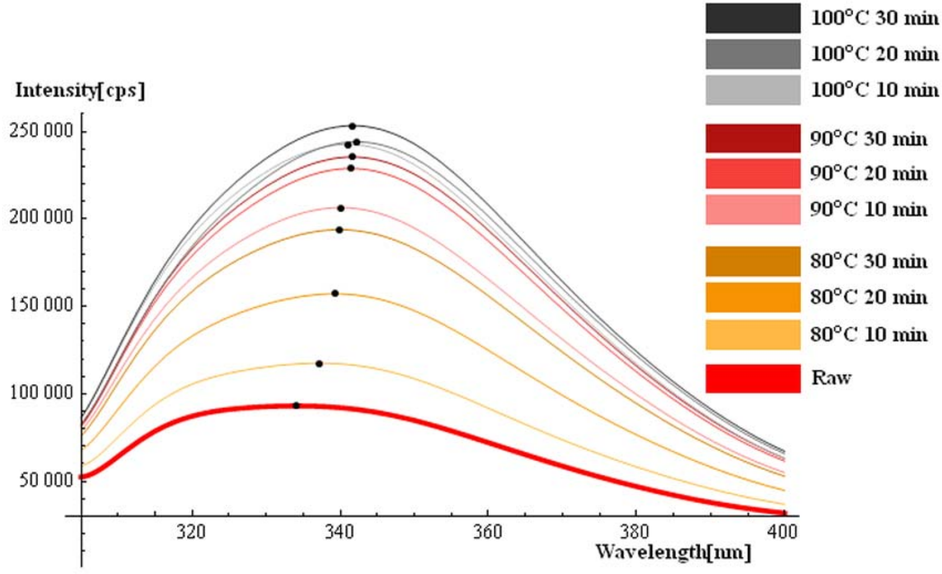


Figure 42. Tryptophan emission spectra of heat treated bovine whey samples

The emission spectra of samples treated at 70°C for different holding times (not shown) were running very close to the spectrum of the control sample. The first big upward turn in intensity was recognized between the 80°C/10 mins and 80°C/20 mins samples. The intensity of the Trp emission curves increased between these settings by ~40.000 cps. This phenomenon indicated that β-Lg started to denature at a temperature somewhat below 80°C. That corresponds well to literature data, where 78 °C was found as denaturation temperature of β-Lg (in phosphate buffer, pH=6,0) (de Wit, Klarenbeek, 1984). The intensity increased significantly between the 80°C/20 mins processed sample and the 80°C/30 mins processed one. Above the 80°C/30 mins treatment conditions the degree of increase in the intensity was getting smaller and smaller, suggesting, that the proportion of whey proteins left to be denatured was getting smaller and smaller.

The different sample sets were compared by t-test. Table 9. shows the p-values.

Table 9. p-values of Trp emission of bovine whey treated either by high pressure or by heat

Treatments compared	**p-values**	**Treatments compared**	**p-values**
Control – 70 °C	0,031324*	Control–200 MPa	0,024398*
70 °C – 80 °C	7,20133 E-63**	200 MPa–600 MPa	0,479059
80 °C – 90 °C	2,29141 E-25**		
90 °C – 100 °C	0,002578**		

* 95% probability level

** 99% probability level

With the exception of pressure treatments, the differences were significant between the sample-set pairs at least at 95% probability level. When the pressurized samples were compared, it was found that pressure increase did not cause significant differences in the Trp emission intensity in whey.

The maxima of the emission spectra are marked. In the spectrum of heat treated whey a clear tendency of red shift could be observed. The wavelength of the emission peak of raw control sample was 334 nm, while as treatment conditions became more severe, the emission shifted to longer wavelengths. The wavelength of the emission peak of 100°C/30 mins sample was found to be 341,7 nm, a 7 nm shift.

Tedford and Schaschke (2000) investigated β-Lg in 0,5 mg/ml, and 2,0 mg/ml concentrations (in bovine milk the concentration of β-Lg is in the order of 3,1 mg/ml). Although they used lower pressures, 55 MPa and 100 MPa, respectively, and pressurized the samples at 35°C and 75°C, respectively, these authors reported, that pressure-temperature treatment at 75°C resulted in an increase in emission wavelength irrespective of pressure. They concluded that structural changes were brought about only by temperature effects, that caused the tryptophan side chains to become more exposed to the surface of the β-Lg molecule, and therefore, to the solvent, indicating an expanded structure.

A possible explanation of the decrease in the intensity of Trp emission of pressurized samples could be, that one of the differences between native and HHP treated protein structure is, that the region rich in tryptophan in the hydrophobic part of the protein gets closer to the core as an effect of HHP, and is shielded from the environment. During high pressure processing cavities inside the protein are filled off, or the protein is so heavily compressed that the gaps disappear. It is resulting in a loss of the protein's functional abilities, but also in a stabilisation of the hydrophobic regions. This might be the reason for the loss in the intensity of the tryptophan emission spectra in the pressurized samples.

With regard to their fluorescence intensity, whey proteins reacted in the opposite way to heat than to pressure. Mills (1976) found that at 20 °C degrees both tryptophan containing regions of β-Lg are in hydrophobic environments. As the temperature is raised, the conformational changes are such, that between 73 °C and 78 °C one of the Trps is transferred to a more polar environment accessible to solvent. Above 78 °C the second Trp residue becomes exposed to solvent. Complete exposure of one residue occurs at 80 °C, while the other one remains partially buried even at 90 °C. Pulgarin, (2005) found, that denaturation of β-lactoglobulin involves the dissociation of a dimer to a monomer, along with changes in the conformation of the polypeptide chain. The change in conformation is a result of disruption of both internal hydrophobic bonds and salt bridges. Based on these findings, the summarized result was that the hydrophobic regions containing tryptophan were loosing their shielding effect and that tryptophan was released gradually to the environment.

7.3.2 Tryptophan Emission Spectra of Bovine Milk

7.3.2.1 Effect of High Pressure on Tryptophan Emission Spectra of Whole Milk

After the graphs of the Trp emission spectra of whole bovine milk were analyzed, the same tendency was found with regard to whey, namely, with increasing pressure the intensity of Trp emission was decreasing. The intensity level was about twice as high in milk samples as in whey samples, while the emission intensity of the HHP processed milk samples decreased more significantly in milk than in whey. Fig. 43. shows the results.

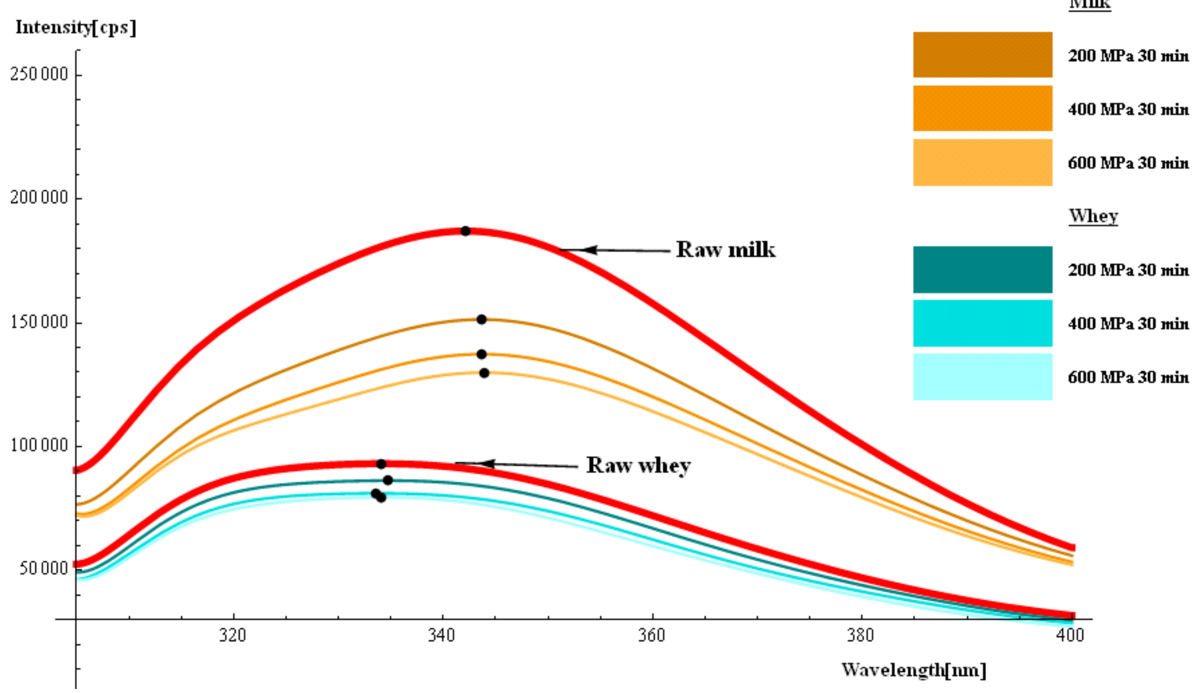


Figure 43. Tryptophan emission spectra of raw and pressurized bovine milk and whey

In bovine milk 80 % of total protein content consists of caseins, and in whey there are practically no caseins, which facilitates the analysing of Trp emission. The spectra were evaluated and the curve spacing was calculated. The differences between the curves of emission spectra were 4-5 times higher in the presence of caseins. The highest intensity level (187.070 cps) among milk samples was that of raw milk. The biggest difference in intensity was 35.719 cps, recorded between the raw and the 200 MPa/30 mins processed sample. In contrast to whole milk, the intensity maximum of emission in control whey was found at 93.095 cps, i.e. ~50% maximum of milk. The greatest deviation (6.839 cps) was obtained again between the control and 200 MPa/30 min processed whey samples.

As already mentioned above, no clear tendency of red shift could be observed in pressurized whey samples, but in whole milk this tendency was clearly apparent. The wavelength corresponding to the emission maximum was 342,13 nm in control milk vs. and 343, 87 nm in the 600/30mins sample, indicating a 1,74 nm shift toward the longer wavelength.

Trp emission peaks were found around 334 nm in whey, and around 343 nm in milk. In proteins, the emission maximum of Trp is found to cover a range of wavelengths from 320 nm (azurin) to 355 nm (albumin) (Weber, 1987), thus the results fit well into the given wavelength range. However, according to Pulgarin’s (2005) measurements, the Trp emission peak of raw bovine milk was detected at 331 nm, which is closer to our results with whey than with whole milk. He hasn’t found any differences between the wavelengths belonging to emission peaks of milk and of whey. Dufour et al. (1997) reported that the maximum of Trp emission in raw bovine milk was at 333 nm.

The difference in the shape of the emission curves of whey and milk is quite noticeable in Fig. 43. The shape of the emission curve in whey was probably due to interference with another fluorescent compound, presumably tyrosine, the presence of which couldn’t be detected in the present measurements. If higher sample dilution had been used, the resolution could have been better, and the reason of the modified spectrum form could have been cleared.

7.3.2.2 Comparison of the Effect of Heat and Pressure Treatment on Milk

Milk proteins reacted in a different way to high pressure processing or heat treatment, respectively. Fig. 44. displays the results.

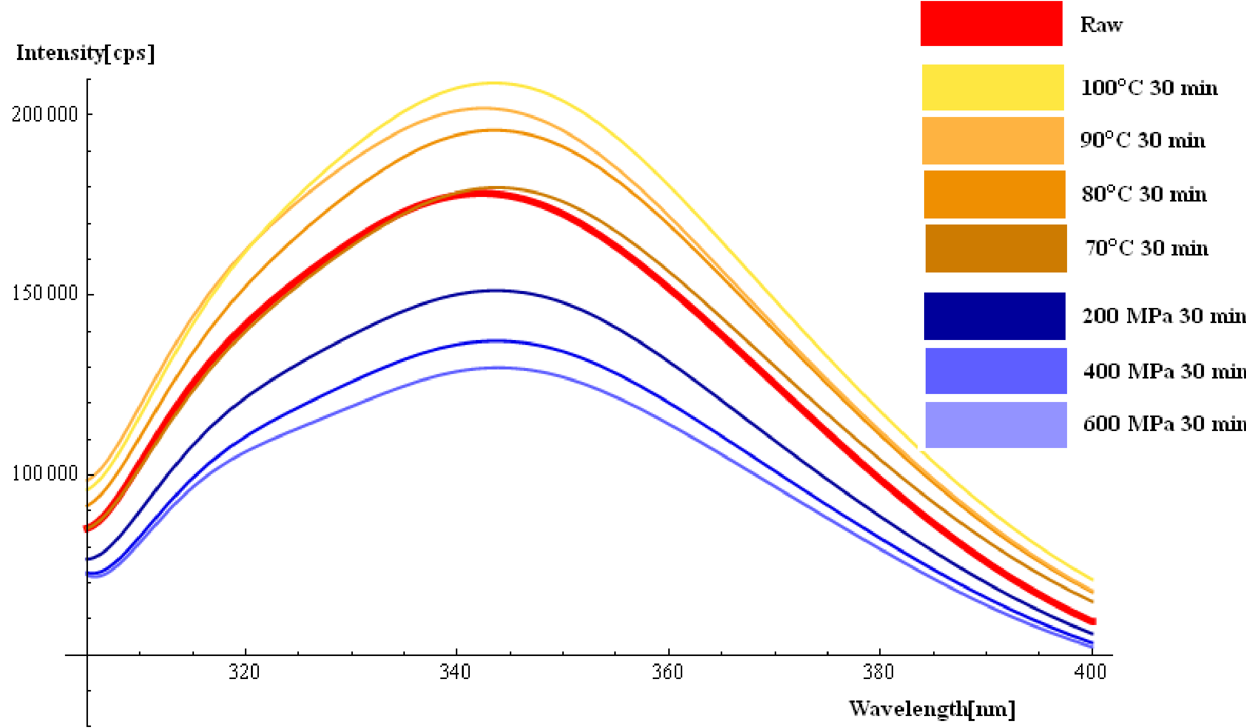


Figure 44. Tryptophan emission spectra of pressurized and heat treated bovine milk

To avoid a confusingly "overcrowded" graph, Fig. 44. shows the Trp emission curves of heat treated and high pressure processed samples only with a holding time of 30 minutes. The intensity of Trp emission spectra increased gradually as heat treatment conditions became more severe. On the other hand, compared to the control samples, HHP processed samples showed a decrease in the intensity of the emission curves, and the differences from the control sample were much smaller than the differences in case of the heat treated ones. For example, the interval between the 600 MPa/30min treated sample and the control sample was 17.245 cps, while the intensity of the 100 °C/30 min treated sample was by 58.945 cps higher than the control sample's.

Results of t-test are shown in Table 10.

Table 10. p-values of Trp emission of bovine milk treated either by high pressure or by heat

Treatments compared	p-values	Treatments compared	p-values
Control – 70 °C	0,145044	Control – 200 MPa	3,8863 E-07**
70 °C – 80 °C	0,001576**	200 MPa–400 MPa	0,417234
80 °C – 90 °C	0,005780**	400 MPa–600 MPa	0,198846
90 °C – 100 °C	0,923985		

* 95 % probability level

** 99% probability level

Significant differences ($p<0,01$) were noted between the following sample-set pairs: samples heated at 70 °C and 80 °C; heated at 80 °C and 90 °C; control (raw) milk and at 200 MPa treated samples. Again, no significant differences appeared between the Trp emission intensities of the pressurized samples. Heating at 90 °C or 100 °C caused almost no differences in the Trp emission intensity of the milk samples.

The wavelength of the emission maximum was shifted by ~1 nm from 342,3 nm to 343,5 nm for the heat treated, and to 343,9 nm for the pressurized samples.

For whey proteins, mainly represented by β-Lg, heat treatment caused the native protein to unfold and to change to denatured state. This resulted from exposure of the hydrophobic regions within the tryptophan residues and agglomeration of the protein causing a loss of screening effects.

When subjected to high pressure treatment, the whey proteins were so heavily compressed or refolded and coagulated, that the Trp containing regions in the hydrophobic part of the protein were forced closer to the core and shielded from the environment.

7.3.2.3 Effect of Cold Storage on Tryptophan Emission Spectra of Milk

In the first series of examinations the samples were cooled immediately after heat treatment to 4 °C to stop the process and fluorescence was measured directly afterwards. In the second series of examinations the samples were cooled, and stored at 4 °C for 18 hours until fluorescence measurement. Differences appeared in the intensities of stored, and directly after processing measured samples (Fig. 45.).

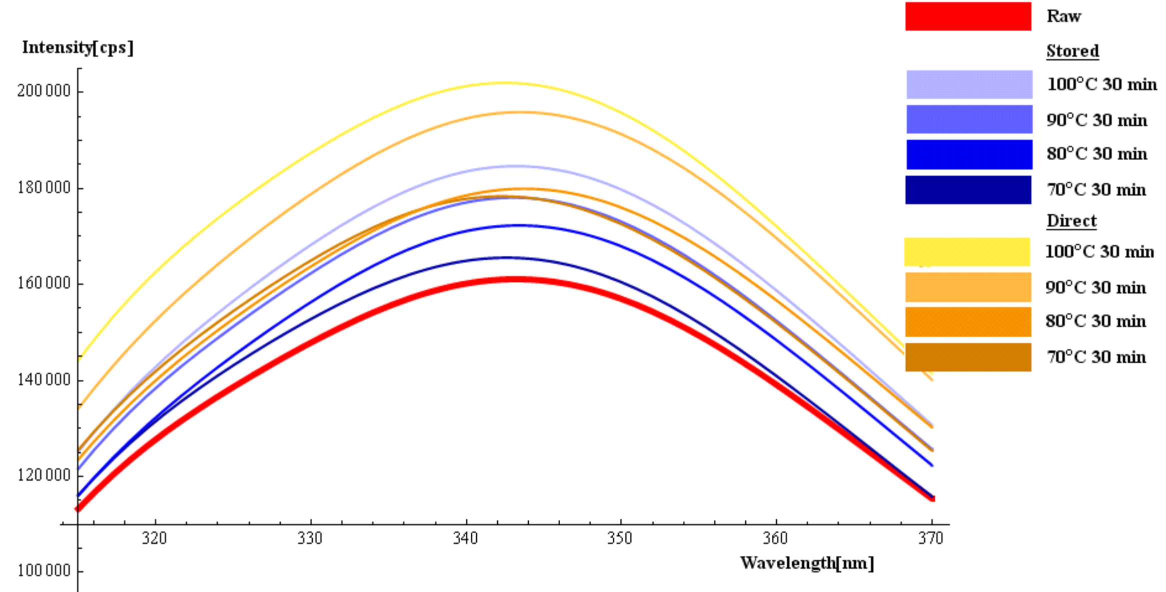


Figure 45. Tryptophan emission spectra of heat treated bovine milk samples measured directly, and after 18 h storage, following treatment

Figure 45. clearly shows, that the Trp emission intensity of those milk samples, that were measured directly after heat treatment, was higher (orange lines), than the intensity of samples, that were cold stored for almost one day after treatment and then measured (blue lines). Not only the intensity, but also the intervals between the spectral curves of stored samples were smaller than those of the "fresh" samples. The emission curve of the 70°C/30 min sample was almost the same as the curve of the 90°C/30 min stored sample, so the structural re-arrangement taking place during storage was equivalent to conformational changes caused by about 20°C drop in temperature in 30 minutes.

This indicated partial refolding of the milk proteins, first of all β-Lg during storage.

Bhattacharjee and Das (2005) studied conformational features of β-Lg. They tracked the intrinsic fluorescence of β-Lg isolate in the course of heating to 90°C and cooling to 25°C. The authors found, that even at 85 °C – 90 °C, β-Lg did not completely lose its folded structure. The unfolding and refolding of β-Lg, as observed by Trp fluorescence, was nearly reversible because the native β-Lg, and its refolded form, following heating and cooling, showed nearly identical Trp emission intensities. However, the findings of Bhattacharjee and Das (2005) did not agree with our results, since in their measurements the emission intensity of Trp was decreasing with increasing temperature.

7.3.3 Tryptophan Fluorescence Emission of Bovine and Goat Milk as Affected by Heat, and HHP Treatment

Goat milk was also included in our investigations. It's fluorescence behaviour affected by heat and HHP treatment, respectively, was studied, and compared to fluorescence behaviour of bovine milk. Fig. 46. shows clearly that the intensity of Trp emission of raw goat milk was higher than that of raw bovine milk (emission spectra highlighted by red). This finding agreed well with the results of Pulgarin et al. (2005), who measured higher Trp emission in whole raw goat milk than in whole raw bovine milk. (Although human milk and ewe's milk were not included in our fluorescence examinations, Pulgarin and co-workers reported that ewe's milk showed the highest emission intensity, followed by goat milk, bovine milk, and human milk which was characterised by weak fluorescence.)

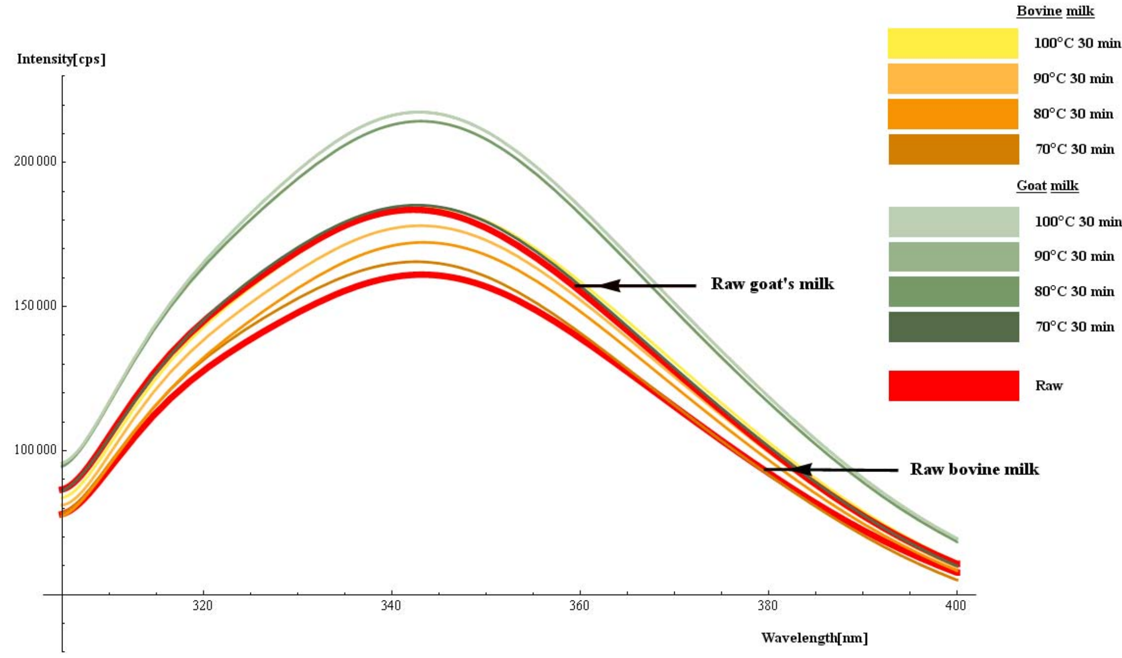


Figure 46. Tryptophan emission spectra of heat treated goat and bovine milk

The tendency in heat treated samples proved to be the same in goat milk as in bovine milk. The intensity of Trp emission increased with increasing temperature and holding time. Analysing the Trp fluorescence spectra of heat treated goat and bovine milk (Fig. 46.), it became evident that goat milk was more sensitive to heat than bovine milk, because its emission intensity increased in a slightly higher degree, than that of bovine milk. The interval between raw and 100°C/30 mins bovine milk sample was 30.698 cps, while between the correlating samples of goat milk this value was 33.897 cps. In the Trp emission intensity of goat milk the differences were not significant between the sample pairs control and heated at 70 °C, and the sample pairs heated at 90 °C and 100 °C. In all the other cases the differences were significant at least at 95% probability level. About 1 nm red shift could be noticed in goat milk samples as an effect of heat treatment.

Raynal and Florent (1998) indicated that pH, micelle hydration, genetic polymorphism of α-casein, non-protein nitrogen, salt balance and ionic calcium could be directly or indirectly involved in the heat sensitivity of goat and bovine milk. But this couldn’t be the only explanation for the different fluorescent intensity levels.

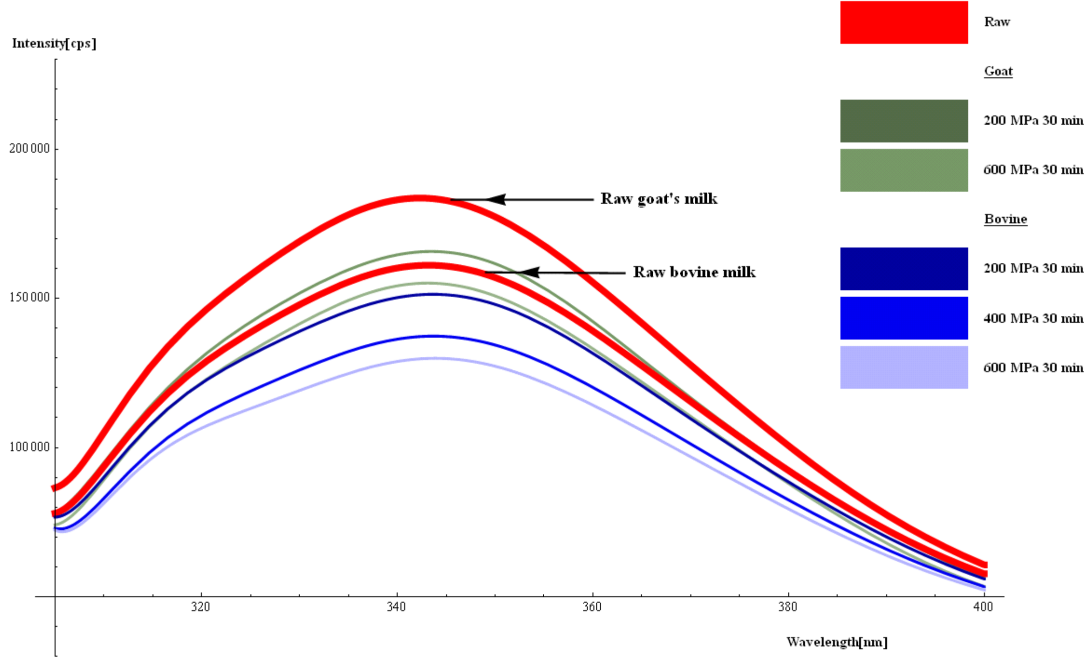


Figure 47. Tryptophan emission spectra of pressurized goat's and bovine milk

Regarding the Trp emission spectra of pressurized goat milk samples, the intensity decreased with increasing pressure and holding time, similarly as in the case of bovine milk (Fig. 47.). The intensity values of Trp emission curves, caused by the same HHP treatment conditions, were found 14.000-25.000 cps lower in bovine milk than in goat milk. In goat milk, the changes in Trp emission intensity caused by the different pressure levels were significant. The rate of decrease was similar in both milk types.

The wavelength of the emission peak in raw goat milk was detected at 342,3 nm, and at 343 nm in the sample pressurized at 600 MPa for 30 mins, that means a red shift smaller than 1 nm.

7.3.4 Effects of High Pressure and Heat Processing on Fluorescence of Retinol in Milk

Another natural fluorescent compound in milk is retinol, the generally available form of retinol in milk. Retinol has a blue-green fluorescence with an excitation maximum of around 330 nm. The most significant feature of the structure of retinol is the conjugated chain of five double bonds (Deshpande, 2001).

7.3.4.1 Effect of High Pressure and Heat on Emission and Excitation Spectra of Retinol in Whole Bovine Milk

The excitation spectra were recorded between 260 nm and 350 nm, and the emission spectra between 350 nm and 500 nm. The emission and excitation spectra of retinol of the HHP processed, and the heat treated bovine milk samples are shown in Fig. 48. and Fig. 49.

Similarly to Trp emission, the emission intensity of retinol in bovine milk showed the tendency to increase with increasing temperature and holding time (not shown), and to decrease with increasing pressure and holding time (not shown).

The emission intensity of heat treated samples increased by 18,5%, while that of pressurized samples decreased by 11%. Wavelength of emission maximum of raw milk was located at 407 nm, and a slight red shift, in the order of 0,7 nm, was observed in the heat treated samples. In pressurized samples a smaller, 0,2 nm red shift could be detected. Dufour and Riaublanc (1997) found the emission maximum in bovine milk at 412 nm.

The differences in the degree of intensity changes, and in the measure of red shift, indicate that the applying pressure affected milk, first of all milk fat, to a lesser extent than applying heat.

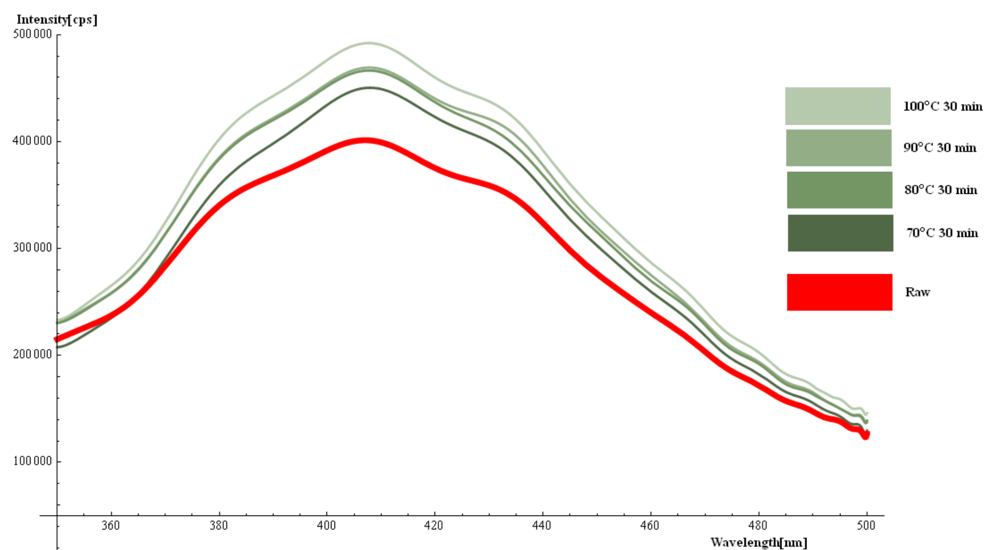


Figure 48. Emission spectra of retinol in heat treated bovine milk

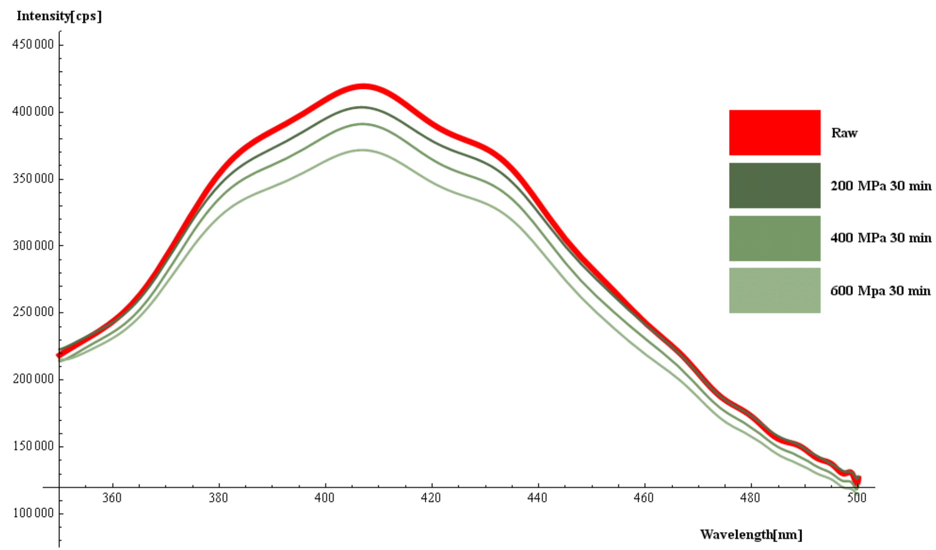


Figure 49. Emission spectra of retinol in pressurized bovine milk

The biggest step was observed between the intensity of raw, and the 70°C/30 mins samples. Among pressurized samples, 400MPa/30 mins and 600MPa/30 mins differed from each other the most. Results of the mathematical statistical analysis (Table 11.) confirm the above statements.

Table 11. p-values of retinol emission of bovine milk treated either by high pressure or by heat

Treatments compared	p-values	Treatments compared	p-values
Control – 70 °C	6,88945 E-16**	Control–200 MPa	0,229751
70 °C – 80 °C	0,001932**	200 MPa–400 MPa	0,841695
80 °C – 90 °C	0,165478	400 MPa–600 MPa	0,007362**
90 °C – 100 °C	6,52759 E-15**		

* 95 % probability level

** 99% probability level

The least changes were caused in retinol emission intensity by increasing the pressure from 200 MPa to 400 MPa. Two hundred MPa pressure as well as rising the temperature from 80 °C to 90 °C did not cause significant changes in the emission intensity of retinol in milk.

Excitation spectra of retinol in heat treated bovine milk, and pressurized bovine milk are presented in Fig. 50. and 51.

The spectra had a characteristic shape with the maxima and two shoulders. The shapes of the spectra were overall similar, varying mainly in the maximum:shoulder intensity ratios. The excitation maximum of raw bovine milk was located at 319 nm, and the shoulder near the maximum at 306 nm. The maxima and the position of the shoulders near the maxima haven’t changed, regardless of the type of treatment. Dufour and Riaublanc (1997) reported similar results. The authors found the maximum of raw bovine milk at 322 nm, and the shoulder at 308 nm.

Fluorescence intensity of heat treated samples increased at higher temperatures, and decreased at higher pressures in the pressurized samples. Thus, the same tendency appeared in these experiments as in the previus measurements.

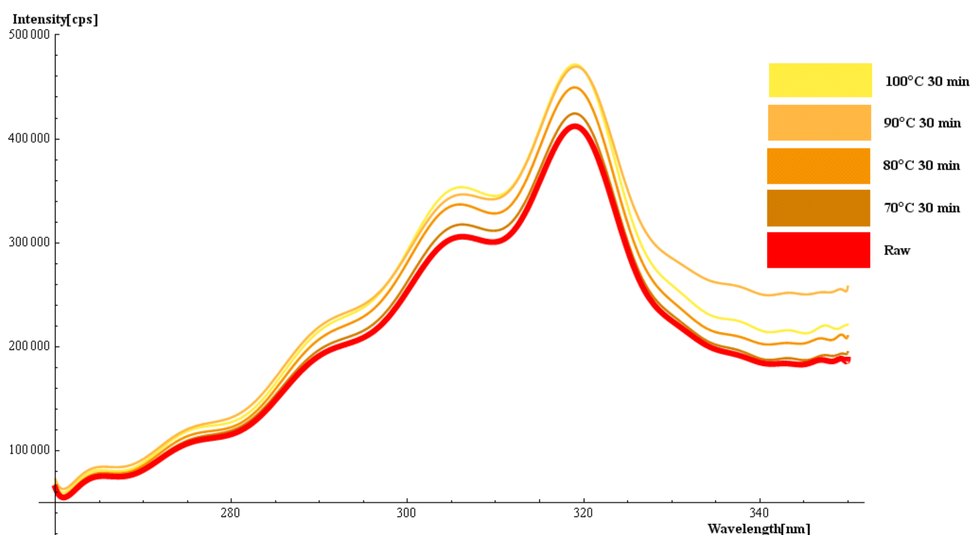


Figure 50. Excitation spectra of retinol in heat treated bovine milk

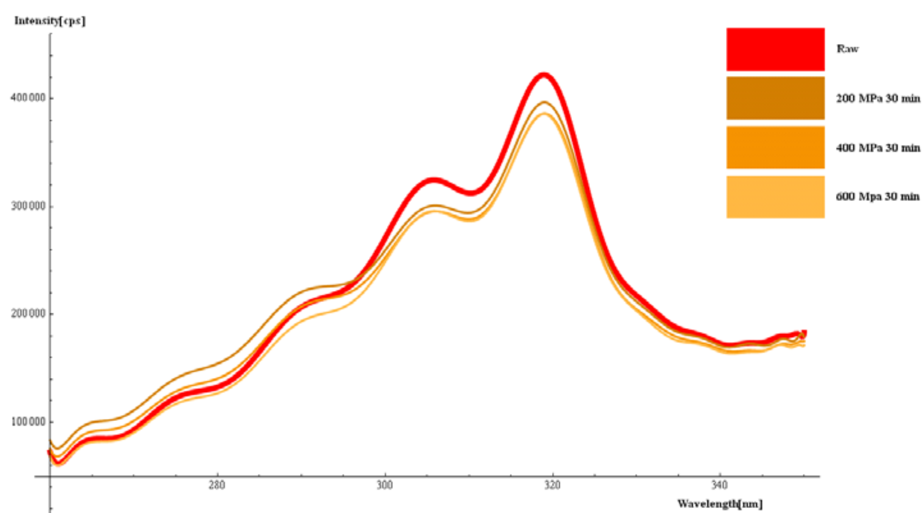


Figure 51. Excitation spectra of retinol in pressurized bovine milk

Intensity of the excitation spectra increased by 13% in the heat treated samples. The biggest difference occurred between the intensity values of the samples heated at 70°C and 80°C. The smallest interval could be observed between the treatments at 90°C and 100°C.

In the pressurized bovine milk samples the decrease in intensity was smaller than in the heat treated ones. Maximum intensity of the sample treated at 600 MPa for 30 mins was 9% lower than that of raw milk. The curves of intensity spectra of 400MPa/30min and 600MPa/30min samples are overlapping each other, showing that increasing pressure from 400 MPa to 600 MPa didn't decrease the fluorescence intensity of retinol.

Table 12. p-values of retinol excitation of bovine milk treated either by high pressure or by heat

Treatments compared	p-values	Treatments compared	p-values
70 °C – 80 °C	0,001173**	200 MPa–400 MPa	0,289177
80 °C – 90 °C	0,137354	400 MPa–600 MPa	0,111824
90 °C – 100 °C	0,001156**		

* 95 % probability level

** 99% probability level

The 200 MPa pressure steps did not cause significant differences in the retinol excitation intensities of the milk samples compared to each other.

7.3.4.2 Effect of High Pressure and Heat on Emission and Excitation Spectra of Retinol in Whole Goat Milk

The emission spectra of retinol in heat treated, and HHP processed goat milk samples are shown in Fig. 52. and Fig. 53.

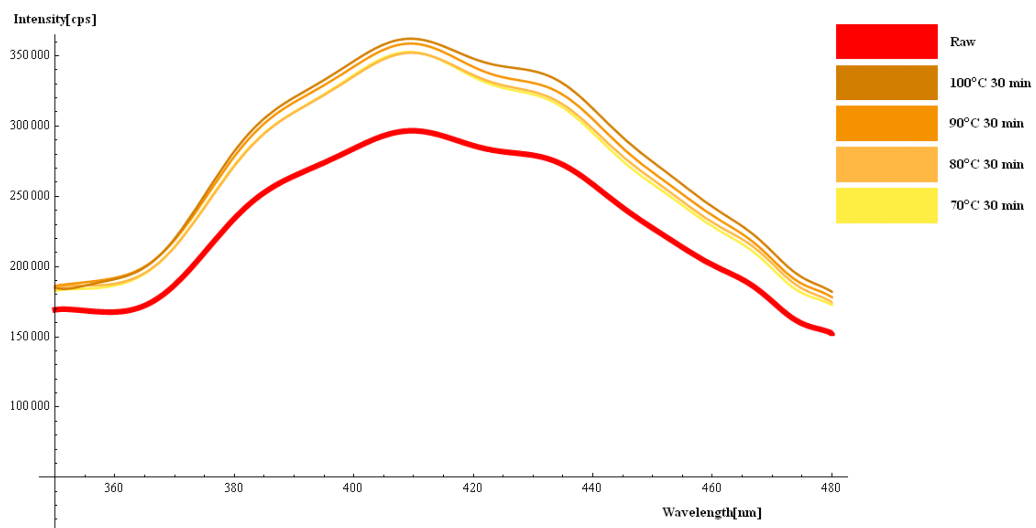


Figure 52. Emission spectra of retinol in heat treated goat milk

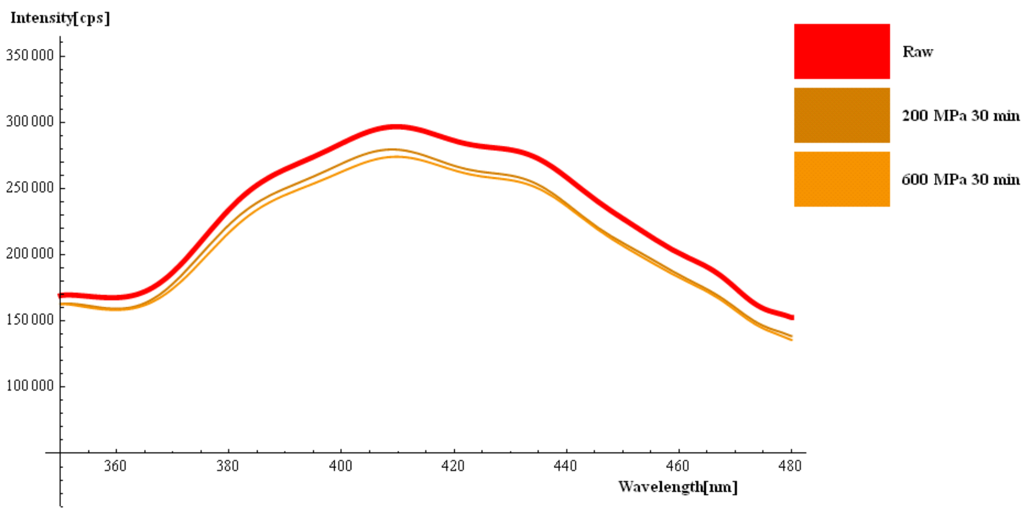


Figure 53. Emission spectra of retinol in HHP treated goat milk

The changes in the intensity of emission spectra of retinol in heat treated goat milk showed the same tendency as the changes in bovine milk. The intensity of emission increased with increasing temperature and holding time (not shown), and decreased with increasing pressure and holding time (not shown). Rate of the changes was higher on heating the milk. The intensity increased by 20% when 100°C/30 mins was applied, compared to the control sample. Differences were significant between the heat treated sample pairs except the sample pairs of 70 °C and 80 °C. In pressurized samples the intensity decreased by 8% as an effect of 600 MPa/30 mins treatment. Two hundred MPa treatment resulted in a significant (99% probability) change in retinol emission compared to the control samples but the application of higher pressure levels did not cause significant changes. The emission wavelength maxima were located at 409,7 nm, and no shift could be observed in either the heated or the pressurized milk batches.

Excitation spectra of retinol in heat, and HHP treated goat milk, are presented in Figures 54. and 55.

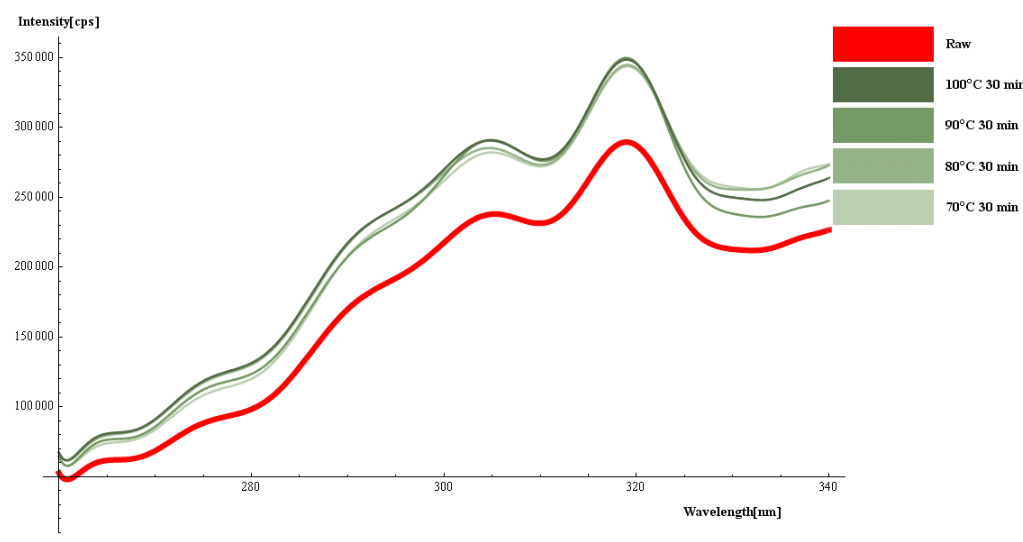


Figure 54. Excitation spectra of retinol in heat treated goat milk

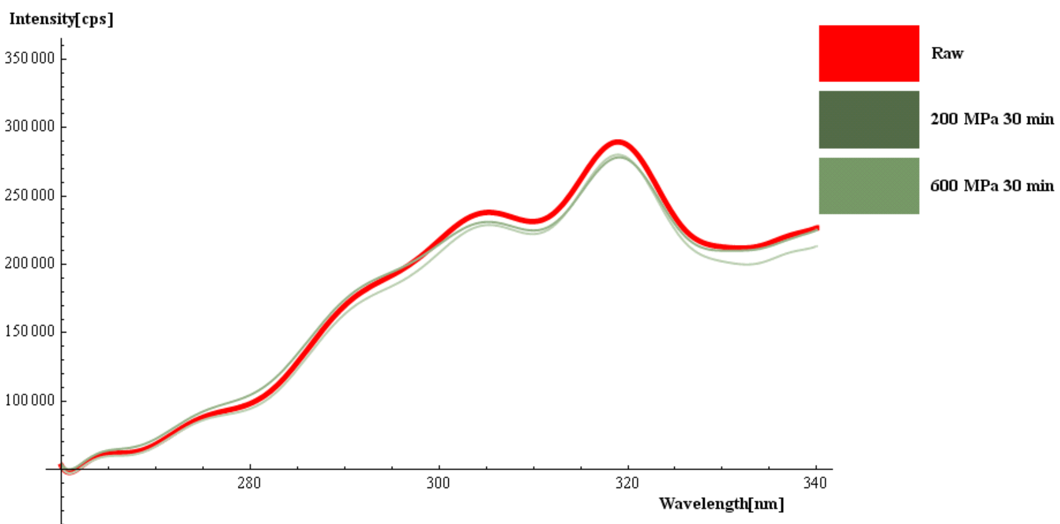


Figure 55. Excitation spectra of retinol in pressurized goat milk

Analysing the excitation spectra of retinol it is apparent again, that heat increased, and pressure decreased the intensity of the spectra. The intensity increased by 16% as an effect of 100°C/30 min treatment, and decreased by a mere 4% as an effect of 600 MPa/30 mins treatment. But the differences in temperature and holding times (not shown), and pressure and holding times (not shown), didn't cause much alteration within the intensities belonging to the matching treatment conditions. The biggest interval was observed always between the raw, and the first sample treated in either way. Results of the paired t-test agree with this statement. Changes were significant (95 % probability) only between the control sample-set and the samples heated at 70 °C and the control sample-set and the samples pressurized to 200 MPa.

Three definite peaks appeared on the spectra. The maxima of the excitation spectra were located at 319 nm, and the shoulder closest to the maximum was found at 305 nm for both types of treatment. No marked shift could be detected, it ranged only within a few decimals.

7.3.4.3 Comparison of the Emission Spectra of Retinol in Bovine and Goat Milk, Respectively

Comparing goat's and bovine milk, both showed similar behaviour under high pressure processing or heat treatment. Nevertheless, the intensity of emission curves was 26 to 28 % higher in bovine milk than in goat milk (Fig. 56., Fig. 57.), that indicated higher retinol content in the examined bovine milk samples than in the goat milk samples.

The wavelength of the emission maximum was at 407 nm for bovine milk, and 409 nm for goat milk.

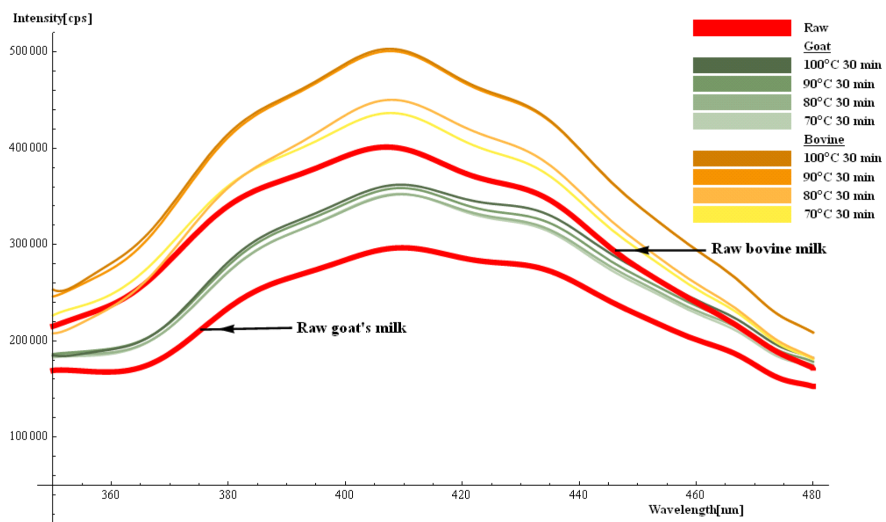


Figure 56. Retinol emission spectra of heat treated goat and bovine milk

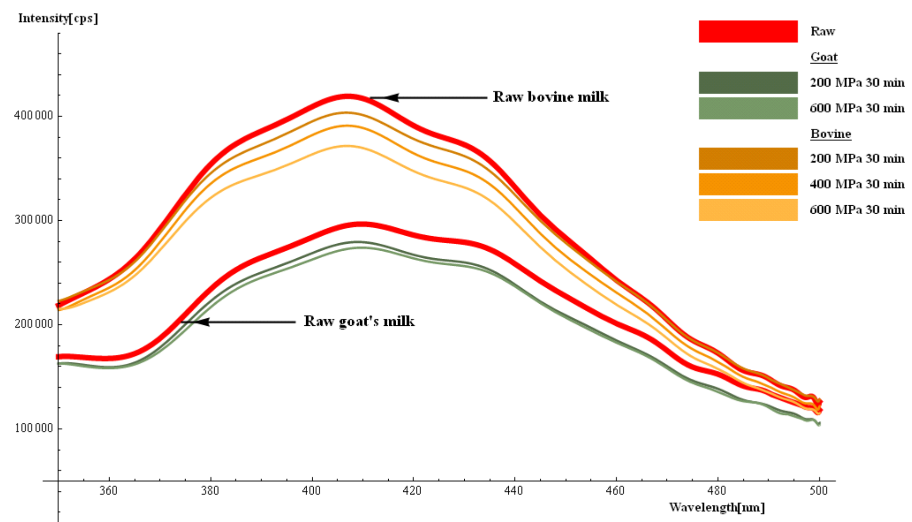


Figure 57. Retinol emission spectra of pressurized goat, and bovine milk

The intensity of the excitation curves as well as the intensity of the emission curves was increasing gradually caused by the release of retinol from the fat globules by heat treatment. Milk fat melts when heated. High pasteurization temperatures denature the cryoglobulins in the fat globule membrane, and aggregation of the fat globules and creaming are impaired or prevented. Severe treatments, 80°C or higher temperature and 15 min or longer holding times, remove lipid and protein materials from the membrane, partially denude the fat globules and may cause them to coalesce and form large clumps of fat (Fox, McSweeney, 1998). Thus retinol, solved in the fat clumps, that have a ruined membrane, was much more exposed to the exciting light, as it was shielded less than in its initial position inside the intact fat globule.

High pressure processing, however, had an opposite effect effect on retinol fluorescence than heat treatment. This phenomenon might have two reasons. One of them is that HHP induced fat crystallisation, and the solid fat content was higher in HHP treated cream, than in the untreated one (Buchheim, Abou El-Nour, 1992; Buchheim et al., 1996a, 1996b). In a solid phase

fluorescence could be less effective. The other reason might be that as the amount of lipolysis products didn't increase in HHP treated milk, HHP did not damage the milk fat globule membrane and so the milk fat globules were not disrupted (Kanno et al., 1998; Ye et al., 2004). Thus, the retinol remained in the intact fat globule and stayed better shielded from the environment. Additionally the fat globules were more compact after the pressure treatment, resulting in a better shielding effect of retinol fluorescence.

As mentioned in the literature overview, β-Lg seem to play an important role in the accumulation of retinol in milk. β-Lg was shown to bind retinol with an apparent association constant similar to that of retinol-binding protein (RBP).

During heat treatment the native structure of β-Lg was denatured. The loss of the secondary, tertiary and quaternary structure of the protein resulted in an irreversible structural change of the central calyx. Therefore the retinol could not bind any longer to the protein, and it was released to the environment. Because of that the denaturation of β-Lg has a synergistic effect on the increase of the emission and excitation intensity of retinol spectra. However, high pressure processing seemed to have less effect on the central calyx. It might be that the EF loop drew the binding site deeper in the protein and so the linking cavity was screened off more effectively.

7.3.5 Mathematical Statistical Comparison of the Two Treatment Methods in the Materials Investigated

Trp emission, retinol emission and excitation intensities were compared by paired t-test in the samples treated at the lowest treatment parameters (200 MPa and 70 °C). Results are presented in Table 13.

Table 13. p-values of different samples treated at 200 MPa and 70 °C, respectively

Comparison of Trp emission intensities at 200 MPa and 70 °C	**p-values**
Bovine milk	0,006366
Bovine whey	0,968963
Goat milk	8,01298 E-07
Comparison of retinol emission intensities at 200 MPa and 70 °C	
Bovine milk	6,44577 E-57
Goat milk	8,38329 E-67
Comparison of retinol excitation intensities at 200 MPa and 70 °C	
Bovine milk	0,000783
Goat milk	1,53337 E-17

Differences caused by heat and pressure were significant (99% probability level) in each case except bovine whey.

7.4 New Scientific Results

1. Higher fat content of milk exerts a protective effect on milk proteins, primarily β-Lg, against pressure. The baroprotective effect of milk fat on milk proteins was confirmed by the examinations on immunoreactivity as well.
2. To my knowledge no research was carried out and published on the treatment of mare’s milk by HHP. Immunoreactivity of mare’s milk was completely eliminated by the application of 600 MPa pressure for 5 mins. Thesis: HHP treated mare’s milk could be a good alternative for patiens suffering from cow’s milk allergy.
3. Immunoreactivities of the two isoforms of β-Lg reacted differently to pressure. Thesis: to decrease the immunoreactivity of β-Lg, pressures higher than 300 MPa are needed.
4. Heat and pressure exerts opposite effects on the tryptophan emission in the materials tested. Intensity of Trp emission increases with increasing temperature and decreases with increasing pressure while maintaining constant holding time. Intensity of Trp emission increases with increasing holding time at constant temperature and decreases with increasing holding time at constant pressure.
5. Heat and pressure exerts opposite effects on the retinol emission and excitation in the materials tested. Intensity of retinol emission and excitation increases with increasing temperature and decreases with increasing pressure while maintaining constant holding time. Intensity of retinol emission and excitation increases with increasing holding time at constant temperature and decreases with increasing holding time at constant pressure.
6. In whole goat milk, the biggest changes in the intensity of retinol emission and excitation take place between the control sample (raw milk) and the samples that were treated to the least extent. Higher treatment parameters cause only very slight differences. Initial changes are the biggest under the applied conditions.

7.5 Új tudományos eredmények

1. Nagyobb zsírtartalmú tej védő hatást gyakorol a nagy nyomással szemben a tejfehérjékre, elsősorban a β-Lg-ra. A nagyobb tejzsír tartalom nyomással szembeni védő hatását az immunreaktivitásra vonatkozó vizsgálatok is alátámasztják.
2. Nyomáskezelés hatására (600 MPa, 5 perc) a kancatej immunreaktiviása teljes mértékben megszűnik. Ily módon a nyomáskezelt kancatej alternatívát jelenthet a tehéntej-fehérje allergiában szenvedők számára.

3. A β-laktoglobulin két izomerje különbözőképpen reagált a nyomáskezelésre. A β-laktoglobulin immunreaktivitásának csökkentéséhez 300 MPa-nál nagyobb nyomásra van szükség.
4. A hőkezelés és a nyomáskezelés ellenkező módon hat a triptofán emisszióra a vizsgált anyagokban. A triptofán emisszió intenzitása a hőkezelés hőmérsékletének emelkedésével nő, a nyomás növelésével csökken állandó tartási idő mellett. A triptofán emisszió intenzitása a tartási idő növelésével állandó hőmérsékleten nő, állandó nyomáson pedig csökken.
5. A hőkezelés és a nyomáskezelés ellenkező módon hat a retinol emissziós és gerjesztési intenzitására a vizsgált anyagokban. A retinol emissziós és gerjesztési intenzitása a hőkezelés hőmérsékletének emelkedésével nő, a nyomás növelésével csökken állandó tartási idő mellett. A retinol emissziós és gerjesztési intenzitása a tartási idő növelésével állandó hőmérsékleten nő, állandó nyomáson pedig csökken.
6. Teljes kecsketejnél a retinol emissziós valamint gerjesztési intenzitásában a legnagyobb arányú változás a kontrol (nyers) és a legkisebb mértékben kezelt minták között van. A nagyobb mértékű kezeléseknél a mért emissziós és gerjesztési intenzitás értékek alig változnak, tehát a kezdeti változások a legnagyobbak.

8 SUMMARY AND CONCLUSIONS

New nutritional concepts require the development and application of technologies which can (1) preserve or improve the overall quality of the raw materials and their physicochemical functionality; (2) maintain or enhance the nutritional and physiological value of the end-product; (3) increase the safety of the product. Minimal processing may be employed successfully to meet these requirements. The goal of the minimal processing concept is to maintain the natural properties of foods and increase the product's shelf life as well as decreasing processing costs and environmental impact, without compromising product safety.

The introduction of the minimal processing concept was made possible by the development and application of novel non-thermal and thermal food processing technologies which are often less invasive than the conventional methods used in the food industry. One of these novel non-thermal techniques is preserving food by high hydrostatic pressure. As this technology has been introduced to food processing only over the last two decades, basic research is still needed to better understand kinetics and mechanisms underlying the effects observed, and more experimental data are required to assure regulatory approvals (Korhonen, 2002).

Prompted by the lack of sufficient data in the field of HHP processing of foods and by the fact that I had access to a lab-scale HHP equipment at the Department of Refrigeration and Livestock Products Technology, my interest was drawn to the potential of this new technology, and I decided to pursue a research project in my field of specialization, dairy science.

The goal of my work was to learn more about the effect of HHP on different types of milk, especially on milk proteins. I used both modern methods of proteomics and spectrofluorometry in the investigations to find out whether the more rapid fluorescence spectroscopy, which is not a conventional method for protein investigations, can provide sufficient information about the changes in milk components compared to the information obtained using the modern methods.

A very important aspect of the introduction of new food processing techniques is whether the new processing method affects the allergenic potential of a food product, since novel foods can be potential allergens. It is necessary to assess the risk of creating or activating hitherto unseen or not bioavailable immunoreactive structures by the application of new food processing technologies. Thus a further objective of my research was to detect the effect of HHP on the immunoreactivity of milk proteins in different milk types.

The following materials were included in the research project: human milk, whole and skimmed bovine milk, whole ewe's milk, whole goat milk, whole mare's milk, and bovine whey.

The samples were treated at different pressure levels and for different holding times. The pressure progressed from 100 MPa to 800 MPa gradually increased by 100 MPa increments, and holding times were 5, 10, 20, 30 and 40 mins. The methods applied in the investigations:

- Polyacrylamide gel electrophoresis (SDS PAGE and native PAGE, two-dimensional PAGE);
- Immunoblotting (Western blotting);
- Spectrofluorometry.

In the spectrofluorometric measurements, whole bovine milk, goat milk and bovine whey were examined. Half of the samples were pressurized and the rest of them underwent heat treatment (from 70°C to 100 °C in 10 °C increments, and from 5 to 30 mins in 5mins increments). The effect of the two preservation methods were compared. Tryptophan emission and retinol emission and excitation intensities were measured. During the tryptophan emission acqusition, wavelength of the excitation was 290 nm and emission was detected between 305 and 450 nm. During the scanning of emission spectra of retinol, intensity of the emitted light was recorded within the wavelength range of 350-500 nm, and the wavelength of excitation was 321 nm. When the excitation spectra of retinol were scanned, the emission wavelength was set to 410 nm, and the excitation wavelengths were recorded between 380 and 600 nm.

In this study, the effect of HHP on different milk types, primarily on their milk proteins, was investigated. In the protein examinations, modern methods used in proteomics, and spectrofluorometry were applied and compared. Investigation of retinol was conducted only by fluorescence spectroscopy. Potential changes in the immunoreactivity of milk proteins as a result of HHP treatment were detected by immunoblotting.

Protein composition of different milk types (human milk, bovine, goat, ewe's and mare's milk) was compared using SDS and 2D PAGE.

In the case of control samples (raw milk), the two albumin milk types were clearly differentiated from the other milk types belonging to the casein milk group. 2D-PAGE gels showed clearly the differences in the amounts of casein fractions between the milk samples. Several spots, indicating the presence of casein, appeared on the gels for goat milk and bovine milk and most of them had higher intensities than spots in the other milk types. As the literature has suggested, no β-Lg was found in human milk. The amount of α-La was less in goat milk and bovine milk than in human or mare's milk samples. In the analysis of ewe's milk, four, rather than two well separated bands appeared on the gels in the position where α-La was expected.

In the next series of examinations, the effect of HHP on different milk proteins was investigated using gel electrophoretic methods. The parameters of pressurization were 600 MPa

and 5 mins holding time. We found that the proteins in milk samples reacted in different ways to pressure treatment.

In human milk, compared to the control sample, very slight or no decrease was observed in the intensity of the casein fractions. A slight decrease was found in the α-La fraction as a result of HHP treatment.

High pressure had slight effect on proteins present in mare's milk. Negligible changes occurred in the intensity of the casein fraction. Intensity of β-Lg increased the most, but not significantly. Intensity of the α-La bands increased merely by ~5%.

Protein fractions of goat milk reacted to HHP treatment in different ways. Among the two peaks of α-La on the densitograms, the first one (lower Rf value) didn't change, while the second one increased notably (approx. 34%). On the other hand, the two peaks corresponding to β-Lg, showed a significant reduction (~55%).

In bovine milk, only a minimal decrease could be observed in the protein fraction of α-La. Intensity of the the two β-Lg bands changed significantly. The rate of decrease was ~50%, close to that of goat milk.

Since in Hungary, compared to other types of milk, production and consumption of bovine milk is of the greatest importance, the effect of HHP on its proteins was investigated in more detail. Bovine milk samples were treated at different pressures (from 100 MPa to 800 MPa) for 10 mins, and for different holding times (5, 10, 20, 30 and 40 mins) at constant pressure, thus the effect of the level of pressure and of the length of holding time could be studied.

Most apparent changes occurred in the β-Lg fraction. According to the intensity of the bands, β-Lg content of pasteurized milk (72°C, 40 s) was approximately the same as the intensity of the sample that had been treated by 300 MPa for 10 mins. By increasing pressure, β-Lg gradually denatured. In the samples pressurized to 800 MPa, this fraction was hardly visible. The bands of proteins, having higher molecular weights, showed an increasingly diffuse distribution indicating aggregation. A small amount (~10%) of native β-Lg remained after HHP treatment at 800 MPa for 20 mins. β-Lg appeared on the gels in two bands representing the two isoforms of this protein. The two isoforms reacted in different ways to pressure, β-Lg B denatured first. In gradient gel, the intensity of casein bands increased in pressurized samples. No significant changes in α-La content of the different pressurized samples could be observed.

Holding time of HHP treatment affected milk proteins as well. The longer the applied treatment time was, the more the intensity of the β-Lg bands decreased. Again, β-Lg B proved to be more sensitive to pressure than β-Lg A. Length of holding time didn't seem to affect significantly the intensities of casein nor of α-La bands based on the separation methods used in this study.

To examine the interactions between proteins and lipids, the patterns of molecular weight separation of proteins were examined, both in control samples and in samples of pressurized skim milk (0.21 g/100g fat content) and whole milk (4.37 g/100g fat content).

Intensity of protein bands changed in a different way in whole and skim milk. Decided differences appeared in the intensities of β-Lg fractions of skim and whole milk samples at 600 and 800 MPa, respectively. The intensity of β-Lg fractions in skim milk decreased more significantly at these pressures than in whole milk. The densitograms showed that a ~4% difference in fat content caused about 40% lower intensity of the β-Lg bands in the skim milk sample at the pressures applied. This suggested a baroprotective effect of fat on proteins. This effect might be explained by the lipid-protein interaction during HHP treatment.

Summarizing the results we found, that intensities of protein fractions in the electrophoretic pattern of HHP treated milk samples decreased with increasing pressure and holding time. The extent of the decrease varied depending on the milk types, and the milk protein fractions reacted to pressure in different ways, too.

In the higher pressure ranges, decrease in the intensity of the protein fractions, first of all of β-Lg, was smaller in the whole milk samples, than in skim milk.

Decrease in the amount of detectable proteins can be explained by the (partial) denaturation/aggregation of milk proteins under HHP. Thus applying HHP can significantly decrease their solubility. Whether the non-thermal, mostly reversible denaturation/aggregation of protein fractions produces advantageous or disadvantageous changes in the conformation and biological activity of milk proteins has yet to be determined.

However, there are no available data on the potential risks of high pressure processing of foods, but it is important to clarify the role of HHP in this respect as well. For this reason we included into our research tests to determine the immunoreactivity of proteins in the control and pressurized milk samples.

In the control samples, immune responses were the strongest in the protein fractions corresponding to casein. Ewe's, goat and bovine milk gave more intensive responses than the other two milk types. β-Lg showed immunoreactivity in each milk of animal origin. The weakest responses were given to α-La by human and mare's milk. In the other three milk types, immunoreactivity caused by this protein fraction could be detected. Two active bands were present. However, when milk positive human serum from an other patient was used in the examinations, the results were different.

After pressure treatment, the most promising results were obtained for mare's and goat milk. We found the least changes in immunoreactivity took place in bovine milk.

Antigen-antibody complexes were investigated in pressurized bovine milk by using anti-β-lactoglobulin antibody IgG developed in rabbit, and human sera for IgE, respectively. Results obtained with the different antibodies were not identical. When anti-β-lactoglobulin antibody IgG was used, no differences were found in the immunoreactivity of casein and α-La fractions in control and pressurized samples. Decrease in immunoreactivity of β-Lg corresponded to the decrease in the intensity of this protein. Three hundred MPa treatment affected β-Lg B in a different way than β-Lg A. At this pressure the intensity of β-Lg B was about half of the original intensity, but β-Lg A showed only a very slight decrease. At 600 MPa the intensity of both β-Lg isoforms showed similar values.

When immunochemical reactions with milk positive human serum were studied, casein fractions gave definite responses. High pressure decreased the immunoreactivity of these fractions, but the rate of decrease reached its maximum at 400 MPa treatment, no further reduction was obtained at higher pressures. The other protein fractions didn't show immunochemical reactions, most likely because the human serum originated from a patient who was sensitive only to casein.

Decrease in immunoreactivity could be noticed only in skim milk but not in whole milk under the applied conditions of the experiment.

HHP seemed to decrease the immunoreactivity of certain protein fractions in the different milk types. According to the separation and immunoblotting methods used, the extent of the decrease was not significant, except for mare's milk. Thus HHP treatment alone did not prove to be useful to produce hypoallergenic milk or milk products.

Heat treated and pressurized bovine milk, bovine whey and goat milk samples were included in the fluorescence investigations. Intensities of tryptophan emission, retinol emission and excitation were measured and compared.

Irrespective of the material investigated and the type of spectra (emission or excitation), the overall tendency was in each case was that the fluorescence intensity increased with higher temperature of the treatment and decreased with increasing pressure.

In the Trp emission measurements, bovine whey showed the lowest intensity values followed by whole bovine milk. Whole goat milk had the highest intensity values. These differences resulted from the composition of the milk types and whey. Whey contains only whey proteins. Casein, the highest protein fraction in milk, can not be found in it. Goat milk contains more protein than bovine milk. The maximum of the emission spectrum of control whey was located at 334 nm and of the 100°C/30 mins sample at 341,7 nm, thus a marked red shift could be observed. More pronounced changes took place in the Trp emission in bovine milk than in whey. The emission peak in raw bovine milk was found at 342 nm. Goat milk had the highest intensity

values and this type of milk reacted to heat and to pressure the most, because its emission intensity changed in a slightly higher degree, than that of bovine milk. About 1 nm red shift could be noticed in goat milk samples as an effect of heat treatment.

Whole bovine milk samples were stored overnight at refrigerator temperature. Intensity of emission spectra of samples measured directly after the treatments were compared to the intensity values of stored samples. Trp emission intensity of stored samples was lower than the intensity of "fresh" samples. Not only the intensity, but also the intervals between the spectral curves of stored samples were smaller. This indicated that structural re-arrangement, primarily partial refolding of milk proteins, first of all β-Lg, took place during storage, and it was equivalent to conformational changes caused by an approx. 20°C drop in temperature.

The tendency, observed in the fluorescence behaviour of Trp under pressure, can be explained as follows. Crystallographic studies have shown that the polarity of Trp environment correlates well with the energy of the fluorescence emission. At higher pressures the native environment of the Trp is replaced by one of considerably greater polarity. Water molecules penetrate the interior of the protein and they cluster close to the Trp residues. Thus strong interaction with the field of the dipole fluorophore becomes possible. Structures of native and HHP treated proteins are different. As an effect of HHP, the Trp containing region in the hydrophobic part of the protein gets closer to the core of the molecule and is shielded from the environment. Cavities inside the protein either are filled off under high pressure, or the protein is so heavily compressed that the gaps disappear. This results in a loss of the protein's functional abilities and in a stabilisation of the hydrophobic regions.

Proteins reacted in the opposite way to heat than to pressure. Structural changes brought about by temperature are such, that the Trp side chains become more exposed to the surface of the protein, primarily β-Lg molecule, and therefore, to the solvent. This indicates an expanded structure. As a result of heat treatment, proteins (partially) unfold and the hydrophobic regions, containing Trp, loose their shielding effect and Trp is released gradually to the environment.

Emission and excitation intensity of retinol was also measured in the two milk types and whey. The same tendencies appeared in this case as in the Trp investigations: the intensity of emission and excitation increased with increasing temperature, and decreased with increasing pressure.

In heat treated bovine milk the maximum difference in retinol emission was observed between the control and the sample heated to 70°C. Among pressurized samples, the biggest interval in intensity was found between the samples subjected to 400 and 600 MPa pressures. When excitation intensities were examined, the maximum difference was registered between the samples processed at 70°C and 80°C, respectively. The biggest difference in excitation

intensities resulted between the control and the sample treated at 200 MPa. The excitation spectra of samples pressurized to 400 and 600 MPa almost overlapped each other, that is, pressures higher than 400 MPa didn't cause any more changes.

Both emission and excitation intensities were lower in goat milk than in bovine milk, due to the smaller retinol content of goat milk. The emission maximum in bovine milk was located at 407 nm and in goat milk at 409 nm. The shape of the excitation spectra differred from the emission spectra. It had one peak and two shoulders at lower wavelengths. The excitation maximum in both milk types was measured at 319 nm. The biggest differences both in emission and excitation intensities were found in goat milk between the control and the sample heated to 70°C, and the control and the sample kept at 200 MPa pressure.

The increase in the intensity of the excitation and emission curves was caused by the release of retinol from the fat globules by heat treatment. High pasteurization temperatures denature the cryoglobulins in the fat globule membrane, and aggregation of the fat globules and creaming are impaired or prevented. Severe heat treatments remove lipids and proteins from the fat globule membrane, partially denude the fat globules and may cause them to coalesce and form large fat clumps. Thus retinol, solved in the fat clumps, with destroyed membranes, is more exposed to the exciting light, since it is shielded less than in its initial position inside the intact fat globule.

High pressure processing had the opposite effect on retinol fluorescence than heat treatment. HHP induced fat crystallisation, and the solid fat content is higher in HHP treated cream and milk, than in the untreated one. Fluorescence shows up less effectively in a solid phase. Besides, the fact that the amount of lipolytic products doesn't increase in HHP treated milk it indicates, that HHP does not damage the milk fat globule membrane and so the milk fat globules are not disrupted Thus the retinol remains in the fat globule and stays better shielded from the environment. Additionally, the fat globules were more compact after the pressure treatment, resulting in a better shielding effect of retinol fluorescence.

β-Lg seem to play an important role in the accumulation of retinol in milk. β-Lg was shown to bind retinol. During heat treatment the native structure of β-Lg is denatured. The loss of the secondary, tertiary and quaternary structure of the protein can result in an irreversible structural change of the central calyx. Therefore retinol can not bind any longer to the protein, and it is released to the environment. As a result, denaturation of β-Lg has a synergistic effect on the increase of the emission and excitation intensity of retinol fluorescence.

Based on the above results we can state that the differences in the degree of intensity changes, and in the measure of red shift, indicate that applying high pressure affected milk, primarily milk proteins and milk fat, to a lesser extent than applying heat. Thus HHP treatment

of milk, considering its effect on the main components, seems to be a milder processing method, than heat treatment.

The results obtained by the two different techniques, gel electrophoresis and spectrofluorometry, were in good agreement with each other. Using gel electrophoresis, intensity of the bands of the different pressurized milk protein fractions was decreasing with increasing pressure and holding time, indicating a loss in the fractions. In accordance with these results, intensities of Trp and retinol emission and excitation spectra were also decreasing with increasing pressure and holding time. The similar tendencies found by the two different methods support the assumption that spectrofluorometry can be a a viable alternative in protein research. However, the type of information provided by PAGE and spectrofluorometry is different. The methods of proteomics can not be replaced by fluorometric measurements, only in specific aimed situations, but where applicable, fluorescence spectroscopy affords rapid, reliable, well-reproducible results in contrast to the time-consuming electrophoretic methods.

Based on these findings, I would like to pursue my research both by concentrating on the spectrofluorometric approach and by broadening my interest towards the application of HHP to the processing of other dairy products (e.g. fermented products such as yoghurt or cheese. Regarding spectrofluorometry, I endeavor to compile a database of milk by systematically adjusting treatment parameters and by measuring the pertinent fluorescence intensities. With the help of an appropriately large data collection, compiled using mathematical statistical methods, such as principal component analysis and discriminance analysis, identification and classification of an unknown sample would be possible. One could also determine, what kind of treatment was used and whether the product underwent an adequate treatment or not. Thus, spectrofluorometry could be made a new and more efficient method of quality control in the dairy industry. Regarding the application of HHP treatment to dairy products other than milk, there is still much to learn about the effect of HHP on their coagulation, texture, ripening, and functional characteristics of proteins.

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10 APPENDIX

1. *12-20% gradient running gel*

	Running gel	
	12%	**20%**
Acrylamide	2 ml	3,35 ml
1,5 M Tris-HCl	1,25 ml	1,25 ml
SDS	50 µl	50 µl
Distilled water	1,7 ml	0,4 ml
TEMED	1,65 µl	1,65 µl
Persulphate	16,5 µl	16,5 µl
Saccharose	-	0,3 g

2. *15 % Native running gel*

	15%
Akrylamide	4,0 ml
2M Tris-HCl	1,8 ml
Distilled water	2,06 ml
TEMED	6 µl
Persulphate	50 µl

3. *Stacking gel*

	SDS
Akrylamide	1,0 ml
10% SDS	55 µl
0,5 M Tris-HCl	660 µl
Distilled water	3,2 ml
TEMED	6 µl
Persulphate	50 µl

There is no SDS in the stacking gel of native-PAGE:

4. *SDS sample solvent*

Tris	0,189 g
SDS	0,5 g
Distilled water	21,2 ml
Glycerol	2,5 ml
β-mercaptoethanol	2,5 ml

5. *Native sample solvent*

=4 ml Native running gel buffer+800 mg saccharose

6. *Running gel buffer* – calculated for 1000 ml distilled water =

= 3,03 g Tris + 14,4 g Glycin + 1 g SDS

There is no SDS in the running gel buffer of native-PAGE.

7. Tracking dye =

= SDS / Native running gel buffer + bromphenolblue

8. LMW molecular weight standard (Bio-Rad)

Colourless LMW

	Molecular weight (kDa)
Phosphorylase	97.0
Bovine serum albumin	66.0
Ovalbumin	45.0
Carbonic anhidrase	30.0
Trypsin inhibítor	20.1
α-lactalbumin	14.4

Coloured LMW – Low range

	Molecular weight (kDa)
Phosphorylase B	106.904
Bovine serum albumin	93.636
Ovalbumin	52.264
Carbonic anhidrase	37.226
Trypsin inhibítor	28.244
Lysosyme	18.833

9. *PAGE-gel washer* =

= 850 ml distilled water + 50 ml acetic acid + 100 ml ethanol

10. *Dye*

Coomassie Brillant Blue R-250	0,2 g
Distilled water	50 ml
Acetic acid	10 ml
Ethanol	50 ml

11. *Sample dilution for 2D-PAGE in rehydrating solution*

For blue staining → 40 µg protein/strip

For immunoblotting → 30 µg protein/strip

Rehydrating solution= 2.4 g urea + bromphenolblue } ⇨ filled to 5 ml by distilled water

↓

1 ml rehydrating solution

+ 10 mg CHAPS + 3.08 mg DDT

12. *Parameters of the IEF apparatus*

S01	250 V	↗ linear	15 min
S02	4000 V	↗ linear	2 h
S03	4000 V	↗ linear	24000 Vh

13. *Dithiothreitol (DTT)*

Equlibrating solution calculated for 10 ml distilled water

Urea	3.6 g
SDS	0.2 g
Tris-HCl	2.5 ml
Glycerol	2.0 ml

30 mg DTT + 1.5 ml equilibrating solution/strip

14. *Iodo-acetamide*

32.5 mg iodo-acetamide + 1.5 ml equilibrating solution/strip

15. *Towbin buffer* – for 1000 ml distilled water

Tris	23,03
Glycine	14,4
Methyl alcohol	200 ml
10% SDS	10 ml

16. *Washing-incubating buffer* (MIB)=0,05 Tris-HCl – for 1500 ml distilled water

Tris	9,07 g
NaCl	13,14 g
Phenyl-methyl-sulphonyl-fluoride	0,0261 g
Tween-20	750 μl

17. *Fixing buffer =*

= 98 ml MIB + 2 ml glutaraldehyde

18. *Covering buffer=*

= 98 ml MIB + 2 ml Tween-20

19. *PBS solution* – for 500 ml distilled water

NaCl	4,0 g
KCl	0,1 g
$Na_2HPO_4 * 2 H_2O$	1,43 g
KH_2PO_4	0,135 g

20. *Developing substrate solution*

30 mg (= 1 tablet) chloro-naphtol + 10 ml 96% ethyl-alcohol

+

50 ml cold PBS solution + 200 μl 30 % H_2O_2

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